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BIS-039

Case Docket No.

Jc586 U.S. PTO

09/145916

09/02/98

Sir:

Transmitted herewith for filing is the patent application of

Inventor: Michael Simons, Rudiger Volk & Arie Horowitz

For: "STIMULATION OF ANGIOGENESIS VIA ENHANCED ENDOTHELIAL  
EXPRESSION OF SYNDECAN-4 CORE PROTEINS"

☒ Enclosed are:

☒ 19 sheets of drawing.

☐ An assignment of the invention to \_\_\_\_\_

☐ A certified copy of a \_\_\_\_\_ application.

☒ An original power of attorney.

☒ A verified statement to establish small entity status under 37 CFR 1.9  
and 37 CFR 1.27. - Independent Inventors.

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APPLICATION FOR LETTRES PATENT

BE IT KNOWN that Michael Simons, Rudiger Volk, and Arie Horowitz  
have made a new and useful improvement entitled "STIMULATION OF  
ANGIOGENESIS VIA ENHANCED ENDOTHELIAL EXPRESSION OF  
SYNDECAN-4 CORE PROTEINS".

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1. The first group of people who are interested in the results of the study are the researchers themselves. They want to know if the study was successful in achieving its objectives and if the results are consistent with their expectations.

2. The second group of people who are interested in the results of the study are the participants. They want to know if the study was fair and if the results are consistent with their own experiences.

3. The third group of people who are interested in the results of the study are the stakeholders. They want to know if the study was useful and if the results are consistent with their own interests.

4. The fourth group of people who are interested in the results of the study are the general public. They want to know if the study was interesting and if the results are consistent with their own beliefs.

5. The fifth group of people who are interested in the results of the study are the media. They want to know if the study was newsworthy and if the results are consistent with their own reporting.

6. The sixth group of people who are interested in the results of the study are the policymakers. They want to know if the study was relevant and if the results are consistent with their own policies.

7. The seventh group of people who are interested in the results of the study are the educators. They want to know if the study was educational and if the results are consistent with their own teaching.

8. The eighth group of people who are interested in the results of the study are the employers. They want to know if the study was practical and if the results are consistent with their own business.

9. The ninth group of people who are interested in the results of the study are the consumers. They want to know if the study was helpful and if the results are consistent with their own needs.

10. The tenth group of people who are interested in the results of the study are the community. They want to know if the study was beneficial and if the results are consistent with their own values.

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formation or growth takes place in most living tissues and organs in mature adults (such as the myocardium of the living heart) [Folkman, J. and Y. Shing, J. Biol. Chem. 267: 10931-10934 (1992); Folkman, J., Nat. Med. 1: 27-31 (1995); Ware, J.A. and M. Simons, Nature Med. 3: 158-164 (1997)]. Moreover, although regulation of an angiogenetic response in-vivo is a critical part of normal and pathological homeostasis, little is presently known about the control mechanisms for this process. A number of different growth factors and growth factor receptors have been found to be involved in the process of stimulation and maintenance of angiogenetic responses. In addition, a number of extracellular matrix components and cell membrane-associated proteins are thought to be involved in the control mechanisms of angiogenesis. Such proteins include SPARC [Sage et al., J. Cell Biol. 109: 341-356 (1989); Motamed, K. and E.H. Sage, Kidney Int. 51: 1383-1387 (1997)]; thrombospondin 1 and 2 respectively [Folkman, J., Nat. Med. 1: 27-31 (1995); Kyriakides et al., J. Cell Biol. 140: 419-430 (1998)]; and integrins  $\alpha v \beta 5$  and  $\alpha v \beta 3$  [Brooks et al., Science 264: 569-571 (1994); Friedlander et al., Science 270: 1500-1502 (1995)]. However, it is now recognized that a major role is played by heparan-binding growth factors such as basic fibrocyte growth factor (bFGF) and vascular endothelial growth factor (VEGF); and thus the means for potential regulation of angiogenesis involves the extracellular heparan sulfate matrix on the surface of endothelial cells.

Research investigations have shown that heparan sulfate core proteins or proteoglycans mediate both heparin-binding growth factor/receptor interaction at the cell surface; and that accumulation and storage of such growth factors within

the extracellular matrix proper occurs [Vlodavsky et al., Clin. Exp. Metastasis 10: 65 (1992); Olwin, B.B. and A. Rapraeger, J. Cell Biol. 118: 631-639 (1992); Rapraeger, A.C., Curr. Opin. Cell Biol. 5: 844-853 (1993)]. The presence of heparin or heparan sulfate is required for bFGF-dependent activation of cell growth in-vitro [Yayon et al., Cell 64: 841-848 (1991); Rapraeger et al., Science 252: 1705-1708 (1991)]; and the removal of heparan sulfate chains from the cell surface and extracellular matrix by enzymatic digestion greatly impairs bFGF activity and inhibits neovascularization in-vivo [Sasisekharan et al., Proc. Natl. Acad. Sci. USA 91: 1524-1528 (1994)]. Ample scientific evidence now exists which demonstrates that any alteration of heparan sulfate (HS) chain composition on the cell surface or within the extracellular matrix which is initiated by means of an altered synthesis, or a degradation, or a substantive modification of glycosaminoglycan (GAG) chains can meaningful affect the intracellular signaling cascade initiated by the growth factor. The importance of heparan sulfate in growth factor-dependent signaling has become well recognized and established in this field.

Heparan sulfate (HS) chains on the cell surface and within the extracellular matrix are present via binding to a specific category of proteins commonly referred to as "proteoglycans". This category is constituted of several classes of core proteins, each of which serve as acceptors for a different type of glycosaminoglycan (GAG) chains. The GAGs are linear co-polymers of N-acetyl-D-glycosamine [binding heparan sulfate] or N-acetyl-D-galactosamine [binding chondroitin sulfate (CS) chains] and aoidic sugars which are attached to these core

1 proteins via a linking tetrasaccharide moiety. Three major classes of HS-carrying  
2 core proteins are present in living endothelial cells: cell membrane-spanning  
3 syndecans, GPI-linked glypicans, and a secreted perlecan core protein [Rosenberg  
4 et al., J. Clin. Invest. 99: 2062-2070 (1997)]. While the perlecan and glypican  
5 classes carry and bear HS chains almost exclusively, the syndecan core proteins  
6 are capable of carrying both HS and CS chains extracellularly. The appearance of  
7 specific glycosaminoglycan chains (such as HS and/or CS) extracellularly on  
8 protein cores is regulated both by the structure of the particular core protein as  
9 well as via the function of the Golgi apparatus intracellularly in a cell-type specific  
10 manner [Shworak et al., J. Biol. Chem. 269: 21204-21214 (1994)].

11 The syndecan class is composed of four closely related family proteins  
12 (syndecan-1,-2,-3 and -4 respectively) coded for by four different genes in-vivo.  
13 Syndecans-1 and -4 are the most widely studied members of this class and show  
14 expression in a variety of different cell types including epithelial, endothelial, and  
15 vascular smooth muscle cells, although expression in quiescent tissues is at a fairly  
16 low level [Bernfield et al., Annu. Rev. Cell Biol. 8: 365-393 (1992); Kim et al.,  
17 Mol. Biol. Cell 5: 797-805 (1994)]. Syndecan-2 (also known as fibroglycan) is  
18 expressed at high levels in cultured lung and skin fibroblasts, although  
19 immunocytochemically this core protein is barely detectable in most adult tissues.  
20 However, syndecan-3 (also known as N-syndecan) demonstrates a much more  
21 limited pattern of expression, being largely restricted to peripheral nerves and  
22 central nervous system tissues (although high levels of expression are shown in the  
23 neonatal heart) [Carey et al., J. Cell Biol. 117: 191-201 (1992)]. All members of

1 the syndecan class are capable of carrying both HS and CS chains extracellularly,  
2 although most of syndecan-associated biological effects (including regulation of  
3 blood coagulation, cell adhesion, and signal transduction) are largely thought to be  
4 due to the presence of HS chains capable of binding growth factors, or cell  
5 adhesion receptors and other biologically active molecules [Rosenberg et al., J.  
6 Clin. Invest. 99: 2062-2070 (1997)].

7 Curiously, however, very little is presently known about and relatively little  
8 research attention has been paid to the function of the syndecan core proteins in-  
9 situ. Syndecan-1 expression has been observed during development suggesting a  
10 potential role in the epithelial organization of the embryonic ectoderm and in  
11 differential axial patterning of the embryonic mesoderm, as well as in cell  
12 differentiation [Sutherland et al., Development 113: 339-351 (1991); Trautman et  
13 al., Development 111: 213-220 (1991)]. Also, mesenchymal cell growth during  
14 tooth organogenesis is associated with transient induction of syndecan-1 gene  
15 expression [Vainio et al., Dev. Biol. 147: 322-333 (1991)]. Furthermore, in adult  
16 living tissues, expression of syndecan-1 and syndecan-4 proteoglycans increases  
17 within arterial smooth muscle cells after balloon catheter injury [Nikkari et al.,  
18 Am. J. Pathol. 144: 1348-1356 (1994)]; in healing skin wounds [Gallo et al.,  
19 Proc. Natl. Acad. Sci. USA 91: 11035-11039 (1994)]; and in the heart following  
20 myocardial infarction [Li et al., Circ. Res. 81: 785-796 (1997)]. In the latter  
21 instances, the presence of blood-derived macrophages appears necessary for the  
22 induction of syndecan-1 and -4 gene expression. However, the effects of changes  
23 in syndecan expression on cell behavior are presently not well understood. For



example, this core protein is believed to mediate bFGF binding and cell activity. Overexpression of syndecan-1 in 3T3 cells led to inhibition of bFGF-induced growth [Mali *et al.*, *J. Biol. Chem.* **268**: 24215-24222 (1993)]; while in 293T cells, overexpression of syndecan-1 augmented serum-dependent growth [Numa *et al.*, *Cancer Res.* **55**: 4676-4680 (1995)]. Furthermore, syndecan-1 overexpression showed increased inter-cellular adhesion in lymphoid cells [Lebakken *et al.*, *J. Cell Biol.* **132**: 1209-1221 (1996)] while also decreasing the ability of B-lymphocytes to invade collagen gels [Libersbach, B.F. and R.D. Sanderson, *J. Biol. Chem.* **269**: 20013-20019 (1994)]. These ostensibly contradictory findings have merely added to the uncertainty and the disparity of knowledge regarding the effects of syndecan expression.

In comparison, the glypican core protein class is composed of five murine and human members and a *Drosophila* *dally* homologue [Rosenberg *et al.*, *J. Clin. Invest.* **99**: 2062-2070 (1997)]. Unlike syndecans, the glypican members are fully extracellular proteins attached to the cell membrane via a GPI anchor. Only one member of the class, glypican-1, is expressed in endothelial cells. Another unique feature of the glypican class of proteoglycans is that they carry substantially only heparan sulfate (HS) chains [Aviezer *et al.*, *J. Biol. Chem.* **269**: 114-121 (1994)]. Consequently, while little is presently known about the biological function of glypicans, they appear able to stimulate FGF receptor 1 occupancy by bFGF and appear able to promote biological activity for several different FGF family members [Steinfeld *et al.*, *J. Cell Biol.* **133**: 405-416 (1996)].



1 Finally, perlecan is the third and last class of heparan sulfate (HS)-carrying  
2 core proteins. Perlecan is a secreted proteoglycan that also has been implicated in  
3 regulation of bFGF activity [Aviezer *et al.*, *Mol. Cell Biol.* 17: 1938-1946 (1997);  
4 Steinfeld *et al.*, *J. Cell Biol.* 133: 405-416 (1996)]. However, little is known  
5 regarding this basal lamina proteoglycan beyond its interaction with basic fibroblast  
6 growth factor receptor.

7 In sum therefore, it is evident that the quantity and quality of knowledge  
8 presently available regarding glycosaminoglycan (GAG) binding core proteins is  
9 factually incomplete, often presumptive, and in some instance apparently  
10 contradictory. Clearly the role of specific proteoglycans as mediators under  
11 varying conditions is recognized; nevertheless, the mechanisms of action and the  
12 functional activity of the various individual classes of core proteins yet remains to  
13 be elucidated in full. Thus, while the role of proteoglycans in some manner  
14 relates to angiogenesis, there is no evidence or data known to date which clearly  
15 establishes the true functional value of proteoglycans nor which establishes a use  
16 for proteoglycans as a means for stimulating angiogenesis in-situ.

#### 17 18 SUMMARY OF THE INVENTION

19  
20 The present invention has multiple aspects and is definable in multiple  
21 contexts. A first primary aspect and definition provides a prepared DNA segment  
22 for placement in a suitable expression vector and transfection of endothelial cells  
23 in-situ such that overexpression of extracellular matrix heparan sulfate

1 proteoglycan entities subsequently occurs in-situ, said prepared DNA segment  
2 comprising:

3 at least one first DNA sequence coding for the extracellular domain of a  
4 discrete proteoglycan entity that is expressed by a transfected endothelial cell in-  
5 situ, said extracellular domain first DNA sequence specifying the extracellular N-  
6 terminal portion of an expressed proteoglycan entity which is then located at and  
7 extends from the endothelial cell surface and is capable of binding heparan sulfates  
8 to form an extracellular matrix in-situ.

9 at least one second DNA sequence coding for the transmembrane domain of  
10 a discrete proteoglycan entity that is expressed by a transfected endothelial cell in-  
11 situ, said transmembrane domain second DNA sequence specifying the medial  
12 portion of an expressed proteoglycan entity which is then located at and extends  
13 through the endothelial cell membrane and is joined with said extracellular N-  
14 terminal portion of said expressed proteoglycan entity; and

15 at least one third DNA sequence coding for the cytoplasmic domain of the  
16 syndecan-4 molecule in said discrete proteoglycan entity that is expressed by a  
17 transfected endothelial cell in-situ, said syndecan-4 cytoplasmic domain third DNA  
18 sequence specifying the cytoplasmic portion of an expressed proteoglycan entity  
19 which is then present within the cytoplasm of a transfected endothelial cell and is  
20 joined to said transmembrane portion and said extracellular N-terminal portion of  
21 said expressed proteoglycan entity.

1 A second primary aspect and definition provides a constructed expression  
2 vector for transfection of endothelial cells in-situ such that overexpression of  
3 extracellular matrix haparan sulfate proteoglycan entities subsequently occurs in-  
4 situ, said constructed expression vector comprising:

5 a prepared DNA segment comprised of

6 (i) at least one first DNA sequence coding for the extracellular  
7 domain of a discrete proteoglycan entity that is expressed by a transfected  
8 endothelial cell in-situ, said extracellular domain first DNA sequence specifying  
9 the extracellular N-terminal portion of an expressed proteoglycan entity which is  
10 then located at and extends from the endothelial cell surface and is capable of  
11 binding heparan sulfates to form an extracellular matrix in-situ,

12 (ii) at least one second DNA sequence coding for the  
13 transmembrane domain of a discrete proteoglycan entity that is expressed by a  
14 transfected endothelial cell in-situ, said transmembrane domain second DNA  
15 sequence specifying the medial portion of an expressed proteoglycan entity which  
16 is then located at and extends through the endothelial cell membrane and is joined  
17 with said extracellular N-terminal portion of said expressed proteoglycan entity,  
18 and

19 (iii) at least one third DNA sequence coding for the cytoplasmic  
20 domain of the syndecan-4 molecule in said discrete proteoglycan entity that is  
21 expressed by a transfected endothelial cell in-situ, said syndecan-4 cytoplasmic  
22 domain third DNA sequence specifying the cytoplasmic portion of an expressed  
23 proteoglycan entity which is then present within the cytoplasm of a transfected

1 endothelial cell and is joined to said transmembrane portion and said extracellular  
2 N-terminal portion of said expressed proteoglycan entity; and  
3 an expression vector carrying said prepared DNA segment and suitable for  
4 transfection of endothelial cells in-situ.

5  
6 A third primary aspect and definition provides an in-situ transfected  
7 endothelial cell which overexpresses extracellular matrix heparan sulfate  
8 proteoglycans and positions on the proteoglycans at the cell surface, said in-situ  
9 transfected endothelial cell comprising:

10 a viable endothelial cell previously transfected in-situ with a constructed  
11 expression vector such that said transfected endothelial cell overexpresses discrete  
12 extracellular matrix heparan sulfate proteoglycan entities coded for by said vector,  
13 said overexpressed proteoglycan entities being comprised of

14 (i) an extracellular N-terminal portion which is located at and  
15 extends from the transfected endothelial cell surface and which binds heparan  
16 sulfates to form an extracellular matrix in-situ, said extracellular N-terminal  
17 portion being the expressed product of at least one first DNA sequence in the  
18 constructed expression vector coding for the extracellular domain of said  
19 proteoglycan entity expressed by the transfected endothelial cell in-situ,

20 (ii) a transmembrane medial portion which is located at and  
21 extends through the endothelial cell membrane and is joined with said extracellular  
22 N-terminal portion of said proteoglycan entity, said transmembrane medial portion  
23 being the expressed product of at least one second DNA sequence in the

1 constructed expression vector coding for the transmembrane domain of said  
2 proteoglycan entity expressed by the transfected endothelial cell in-situ, and  
3 (iii) a syndecan-4 cytoplasmic portion present within the  
4 cytoplasm of the transfected endothelial cell which is joined to said transmembrane  
5 portion and said extracellular N-terminal portion of said proteoglycan entity, said  
6 syndecan-4 cytoplasmic portion being the expressed product of at least one third  
7 DNA sequence in the constructed expression vector coding for the cytoplasmic  
8 domain of the syndecan-4 molecule of said proteoglycan entity expressed by the  
9 transfected endothelial cell in-situ.

10  
11 A fourth primary aspect and definition provides a method for stimulating  
12 angiogenesis in-situ within a living tissue comprising vascular endothelial cells,  
13 said method comprising the steps of:

14 transfecting vascular endothelial cells within a living tissue with a  
15 constructed expression vector such that the resulting transfected vascular  
16 endothelial cells overexpress discrete extracellular matrix heparan sulfate  
17 proteoglycan entities coded for by said constructed expression vector, said  
18 overexpressed proteoglycan entities being comprised of

19 (i) an extracellular N-terminal portion which is located at and  
20 extends from the transfected vascular endothelial cell surface and binds heparan  
21 sulfates to form an extracellular matrix in-situ, said extracellular N-terminal  
22 portion being the expressed product of at least one first DNA sequence in the  
23 constructed expression vector coding for the extracellular domain of said

1 proteoglycan entity expressed by a transfected vascular endothelial cell in-situ,  
2 (ii) a transmembrane medial portion which is located at and  
3 extends through a transfected vascular endothelial cell membrane and is joined with  
4 said extracellular N-terminal portion of said proteoglycan entity, said  
5 transmembrane medial portion being the expressed product of at least one second  
6 DNA sequence in the constructed expression vector coding for the transmembrane  
7 domain of said proteoglycan entity expressed by a transfected vascular endothelial  
8 cell in-situ, and  
9 (iii) a syndecan-4 cytoplasmic portion present within the  
10 cytoplasm of a transfected vascular endothelial cell which is joined to said  
11 transmembrane portion and said extracellular N-terminal portion of said expressed  
12 proteoglycan entity, said syndecan-4 cytoplasmic portion being the expressed  
13 product of at least one third DNA sequence in the constructed expression vector  
14 coding for the cytoplasmic domain of the syndecan-4 molecule of said proteoglycan  
15 entity expressed by a transfected vascular endothelial cell in-situ; and  
16 allowing said transfected vascular endothelial cells bearing said  
17 overexpressed extracellular matrix proteoglycan entities to stimulate angiogenesis  
18 in-situ.

#### 20 BRIEF DESCRIPTION OF THE FIGURES

21  
22 The present invention can be more easily understood and better appreciated  
23 when taken in conjunction with the accompanying drawing, in which:

1 Fig. 1 is a representation of a prepared DNA sequence fragment;  
2 Fig. 2 is a recitation of the DNA sequence coding for the extracellular  
3 domain of syndecan-1;  
4 Fig. 3 is a recitation of the DNA sequence coding for extracellular domain  
5 of syndecan-2;  
6 Fig. 4 is a recitation of the DNA sequence coding for the extracellular  
7 domain of syndecan-3;  
8 Fig. 5 is a recitation of the DNA sequence coding for the extracellular  
9 domain of syndecan-4;  
10 Fig. 6 is a recitation of the DNA sequence coding for the extracellular  
11 domain of glypican-1;  
12 Fig. 7 is a recitation of the DNA sequence coding for the transmembrane  
13 domain of syndecan-1;  
14 Fig. 8 is a recitation of the DNA sequence coding for the transmembrane  
15 domain of syndecan-2;  
16 Fig. 9 is a recitation of the DNA sequence coding for the transmembrane  
17 domain of syndecan-3;  
18 Fig. 10 is a recitation of the DNA sequence coding for the transmembrane  
19 domain of syndecan-4;  
20 Fig. 11 is a recitation of the DNA sequence coding for the transmembrane  
21 domain of GPI;  
22 Fig. 12 is a recitation of the DNA sequence coding for the transmembrane  
23 domain of perlecan;



Fig. 13 is a recitation of the DNA sequence coding for the cytoplasmic domain of syndecan-4;

Fig. 14 is a graph illustrating the in-vitro growth assays of ECV-derived cell clones;

Figs. 15A-15C are photographs showing the results of Matrigel growths assays;

Fig. 16 is a graph illustrating the effect of syndecan construct expression on endothelial cell migration in Boyden chamber assays;

Figs. 17A-17F are photographs showing BudR uptake in op/op homozygous (-/-) and heterozygous (+/-) mice;

Fig. 18 is a photograph showing Northern blot analysis of gene expression in PR-39 transgenic mice; and

Fig. 19 is a graph illustrating in-vitro microvascular reactivity in PR-39 transgenic mice.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention provides both the tangible means and the methods for causing an overexpression of extracellular, heparan sulfate carrying, proteoglycans on-demand at and through the surface of endothelial cells; and via such on-demand overexpression of proteoglycans to stimulate angiogenesis in-situ. The tangible means include a prepared DNA segment comprising sequences coding for an extracellular domain, a transmembrane domain, and the cytoplasmic domain of the

1 syndecan-4 protein; as well as a constructed expression vector for the transfection  
2 of endothelial cells in-situ such that overexpression of extracellular matrix, heparan  
3 sulfate bearing, proteoglycan entities subsequently occurs in-situ. The resulting  
4 transfected endothelial cell overexpresses proteoglycans and positions them at the  
5 cell surface - thereby providing the structural and functional entities by which to  
6 stimulate angiogenesis in-situ.

7 A number of major benefits and advantages are therefore provided by the  
8 means and methods comprising the present invention. These include the following:

9 1. The present invention provides in-situ stimulation for angiogenesis. By  
10 definition, therefore, both in-vivo and in-vitro circumstances of use and application  
11 are envisioned and expected. Moreover, the endothelial cells which are to be  
12 transfected such that overexpression of proteoglycans subsequently occurs, may be  
13 alternatively isolated endothelial cells, be part of living tissues comprising a variety  
14 of other cells such as fibrocytes and muscle cells, and may also comprise part of  
15 specific organs in the body of a living human or animal subject. While the user  
16 shall choose the specific conditions and circumstances for practicing the present  
17 invention, the intended scope of application and the envisioned utility of the means  
18 and methods described herein apply broadly to living cells, living tissues,  
19 functional organs and systems, as well as the complete living body unit as a viable  
20 whole.

21 2. The present invention has a variety of different applications and uses. Of  
22 clinical and medical interest and value, the present invention provides the  
23 opportunity to stimulate angiogenesis in tissues and organs in a living subject

1 which has suffered defects or has undergone anoxia or infarction. A common  
2 clinical instance is the myocardial infarction or chronic myocardial ischemia of  
3 heart tissue in various zones or areas of a living human subject. The present  
4 invention thus provides opportunity and means for specific site stimulation and  
5 inducement of angiogenesis under controlled conditions. The present invention  
6 also has major research value for research investigators in furthering the quality  
7 and quantity of knowledge regarding the mechanisms controlling angiogenesis  
8 under a variety of different conditions and circumstances.

9 3. The present invention envisions and permits a diverse range of routes of  
10 administration and delivery means for introducing a constructed expression vector  
11 to a specific location, site, tissue, organ, or system in the living body. A variety  
12 of different expression vectors are available to the practitioner; and a diverse and  
13 useful range of delivery systems which are conventionally available and in  
14 accordance with good medical practice are adapted directly for use. In this  
15 manner, not only are the means for transfection under the control of the user, but  
16 also the manner of application and limiting the locale or area of intentional  
17 transfection of endothelial cells can be chosen and controlled.

18 4. The user also has the choice and discretion of the manner in which the  
19 DNA segment is prepared - so long as the prepared DNA fragment conforms to  
20 the minimal requirements set forth herein. Thus, the prepared DNA sequence  
21 fragment may comprise the entire syndecan-4 DNA sequence in each of the  
22 required extracellular, transmembrane, and cytoplasmic domains. However, it is  
23 expected and envisioned that the more frequent choice will be a chimera core

1 protein structure which comprises only the syndecan-4 cytoplasmic domain but  
2 incorporates transmembrane and extracellular domains which are not native to the  
3 DNA of syndecan-4. Thus, the majority of prepared DNA sequenced fragments  
4 will be chimeric DNA segments ligated together intentionally using recombinant  
5 techniques and methods to form a unitary DNA fragment.

6 5. The present invention provides a unique capability and control for  
7 stimulating angiogenesis in-situ by genetic manipulation of the endothelial cells as  
8 they exist within the tissues and organs as found. This level of gene control and  
9 utilization of the expression mechanisms found within the cytoplasm of the  
10 endothelial cells themselves provides a point of intentional intervention which  
11 harnesses and utilizes the cellular systems of the endothelial cells themselves to  
12 produce the intended and desired result. The transfected endothelial cells in-situ  
13 are thus minimally altered from their original genetic constituents; and the  
14 methodology utilizes the natural regulatory and protein producing systems of the  
15 endothelial cells themselves to provide the overexpression of proteoglycans which  
16 are located and positioned in the normally expected manner by the endothelial cells  
17 as part of the normal homeostatic mechanisms.

18 Accordingly, by the very requirements of the present invention it is thus  
19 important, if not essential, that the user be at least familiar with the many  
20 techniques for manipulating and modifying nucleotides and DNA fragments which  
21 have been reported and are today widespread in use and application. Merely  
22 exemplifying the many authoritative texts and published articles presently available  
23 in the literature regarding genes, DNA nucleotide manipulation and the expression

of proteins from manipulated DNA fragments are the following: Gene Probes for Bacteria (Macario and De Marcario, editors) Academic Press Inc., 1990; Genetic Analysis, Principles Scope and Objectives by John R.S. Ficham, Blackwell Science Ltd., 1994; Recombinant DNA Methodology II (Ray Wu, editor), Academic Press, 1995; Molecular Cloning, A Laboratory Manual (Maniatis, Fritsch, and Sambrook, editors), Cold Spring Harbor Laboratory, 1982; PCR (Polymerase Chain Reaction), (Newton and Graham, editors), Bios Scientific Publishers, 1994; and the many references individually cited within each of these publications. All of these published texts are expressly incorporated by reference herein.

In addition, a number of issued U.S. Patents and published patent applications have been issued which describe much of the underlying DNA technology and many of the conventional recombinant practices and techniques for preparing DNA sequences coding for core proteins such as syndecan-4. Merely exemplifying some of the relevant patent literature for this subject are: U.S. Patent Nos. 5,486,599; 5,422,243; 5,654,273; 4,356,270; 4,331,901; 4,273,875; 4,304,863; 4,419,450; 4,362,867; 4,403,036; 4,363,877; as well as Publications Nos. W09534316-A1; W09412162-A1; W09305167-A1; W09012033-A1; W09500633; W09412162; and R09012033. All of these patent literature publications are also expressly incorporated by reference herein.

## I. Constructed Core Protein DNA Fragments

A primary component part of the subject matter as a whole comprising the present invention is the manufacture and proper use of a prepared DNA segment intended for placement in a suitable expression vector; and useful for transfection of endothelial cells in-situ, under both in-vivo and in-vitro conditions, such that overexpression of extracellular matrix heparan sulfate carrying proteoglycans subsequently occurs in-situ. The prepared DNA segment is a manufactured or synthesized nucleotide fragment which preferably exists as a single, coiled strand of DNA bases in series; and constitutes sufficient DNA information to code for three requisite domains as illustrated by Fig. 1.

Fig. 1 is a simplistic and broadly representational illustration of the prepared DNA fragment after manufacture or synthesis. As seen therein, the prepared DNA segment comprises at least a first DNA sequence coding for the extracellular domain of a discrete and identifiable proteoglycan entity which, after being expressed by a transfected endothelial cell in-situ, yields a specified N-terminal portion of an expressed proteoglycan entity. This N-terminal portion is the extracellular region of the expressed proteoglycan molecule which is then located at and extends from the transfected endothelial cell surface. This extended, extracellular N-terminal region (expressed as specific amino acid residues in sequence) is capable of binding heparan sulfates at the cell surface thereby forming an extracellular heparan sulfate matrix in-situ.

1 The prepared DNA segment fragment illustrated by Fig. 1 must also  
2 provide at least one second DNA sequence coding for the transmembrane domain  
3 of a discrete proteoglycan entity that is expressed by a transfected endothelial cell  
4 in-situ. This transmembrane domain second DNA sequence codes for and  
5 specifies the amino acid residue sequence of the medial or central portion of an  
6 expressed proteoglycan entity by the transfected endothelial cell. The medial  
7 portion or central region of the expressed proteoglycan is located at and extends  
8 through the endothelial cell membrane and is directly joined with and to the  
9 extracellular N-terminal portion of the expressed proteoglycan then extending from  
10 the cell surface.

11 The final requisite component of the prepared DNA segment illustrated by  
12 Fig. 1 comprises at least one third DNA sequence coding for the cytoplasmic  
13 domain of the syndecan-4 molecule within the discrete proteoglycan entity that is  
14 expressed by a transfected endothelial cell in-situ. This third DNA sequence  
15 specifies the cytoplasmic domain of the syndecan-4 DNA; and thus requires the  
16 expression of the particular amino acid residues which identify the syndecan-4  
17 cytoplasmic region of the syndecan-4 core protein structure. While some small  
18 variation is permitted within the third DNA sequence specifying the cytoplasmic  
19 domain of the syndecan-4 amino acid structure, it is essential and required in every  
20 embodiment of the prepared DNA fragment which is the present invention that the  
21 expressed cytoplasmic region of the proteoglycan entity then present within the  
22 cytoplasm of a transfected endothelial cell be identifiably recognized as being a  
23 syndecan-4 amino acid residue type. In addition, the expressed cytoplasmic



1 portion constituting the syndecan-4 amino acid sequence must be present within the  
2 cytoplasm of a transfected endothelial cell; and be joined to the transmembrane  
3 portion and the extracellular N-terminal portion of the expressed proteoglycan  
4 entity.

5  
6 The heterogeneous domains joined together as a unitary fragment

7 It will be recognized and appreciated that the prepared DNA sequence is  
8 intended to be primarily, but not always, a heterogeneous DNA structure which  
9 joins together individual and separate DNA sequences as a unitary fragment. The  
10 cytoplasmic domain constituting the third DNA sequence of the prepared fragment  
11 is limited and restricted to those DNA bases in sequence which recognizably and  
12 identifiably code for the syndecan-4 amino acid residues. Although single point or  
13 small variant alternations or modifications in the DNA base sequence is  
14 permissible and expected, the overall domain must be in each and every instance  
15 recognizable and identifiable (using appropriate analytical means) as representative  
16 of the cytoplasmic region of the syndecan-4 molecular structure.

17 In comparison, the practitioner or intended user has the choice of many  
18 different DNA sequences and formats when choosing and selecting DNA sequences  
19 coding for the extracellular domain coding for the N-terminal region and the  
20 transmembrane domain coding for the central or medial region of the proteoglycan  
21 molecule to be expressed. Thus, the user may construct the entirety of the  
22 syndecan-4 DNA base sequence in its entirety such that a complete syndecan-4  
23 core protein is subsequently expressed by a transfected endothelial cell. However,

1 it is expected that in many instances the heterogeneous combination of individual  
2 and separate DNA base sequences representative of other and different core protein  
3 structures will be utilized; and that the resulting expressed proteoglycan entity will  
4 therefore be a chimeric core protein having different amino acid residues  
5 constituting the transmembrane region and the extracellular region of the expressed  
6 proteoglycan entity. Thus it is expected and envisioned that the first DNA  
7 sequence may be the DNA coding for the glypican-1 amino acid residues; while  
8 the second DNA sequence coding for the transmembrane domain may be  
9 representative of the syndecan-1 amino acid structure. Thus, the availability and  
10 use of heterogeneous prepared DNA fragments linking together first, second, and  
11 third DNA sequences - each of which is representative of a different core protein  
12 content and structure - thus will yield the expression of a chimeric proteoglycan  
13 entity which does not and cannot occur in nature.

14 In addition, the present availability of manufacturing heterogeneous DNA  
15 fragments which will yield an expressed chimera core protein in a transfected  
16 endothelial cell in-situ allows the intended user to choose and more carefully align  
17 the amino acid composition of the expressed proteoglycan entity to be in  
18 accordance with and more compatible to the particular clinical problem and  
19 specific living tissue which is the intended treatment target. Thus, if damaged  
20 myocardium is the tissue intended as the target for treatment, the manufacture of  
21 the heterogeneous fragment might include an extracellular domain (the first DNA  
22 sequence) coding for the glypican-1 region; which is joined to the transmembrane  
23 DNA domain (the second DNA sequence) which itself codes for a syndecan-2

1 amino acid region; which in turn is linked to the cytoplasmic domain (the third  
2 DNA sequence) which must code for the syndecan-4 region. In comparison,  
3 however, if the targeted tissue is lung tissue, the extracellular domain might be  
4 representative of the syndecan-1 amino acid region; while the transmembrane  
5 domain represents the DNA coding for the amino acids of the syndecan-3 region;  
6 and the cytoplasmic domain continues to code exclusively for the syndecan-4  
7 region. In other words, the extracellular domain can be specifically tailored to an  
8 environment where it will be expressed.

9 In this manner, the manufacturer or intended user may customize and tailor  
10 the DNA sequences constituting the extracellular domain and/or the transmembrane  
11 domain as far as possible to best meet or suit the particular tissue, clinical  
12 condition, or pathology then existing and critical to the particular application of  
13 interest. The range and variety of choices, therefore, allows the manufacturer and  
14 intended user a greater degree of flexibility, of potential therapeutic effects, and a  
15 greater degree of individuality than has ever been possible before the present  
16 invention was made.

#### 17 18 Manufacture of the prepared DNA sequence fragment

19 It is expected and intended that the conventionally known and commonly  
20 used recombinant DNA materials, procedures, and instrumentation will be  
21 employed for the manufacture of the prepared DNA sequence fragments. Thus,  
22 the entire prepared DNA sequence structure including the entirety of the  
23 extracellular domain and the transmembrane domain, and the cytoplasmic domain

1 coding for the syndecan-4 structure may be synthesized directly from individual  
2 bases using the commercially available instruments and techniques. Alternatively,  
3 the DNA sequences existing in naturally occurring core proteins may be replicated;  
4 and the cDNA isolated from individual clones using the appropriate enzymes and  
5 protocols. Regardless of the methods and means of manufacture, any and all of  
6 these protocols, procedures, systems, or instruments which will yield the prepared  
7 DNA sequence as an discrete fragment is suitable and appropriate for use with the  
8 present invention.

9 A preferred technique, procedure, and methodology for preparing the DNA  
10 fragment as a whole is given in the Materials and Methods portion of the  
11 Experiments presented hereinafter. The described method, however, is merely one  
12 among many conventionally known and available for this purpose.

#### 13 14 A. The Extracellular Domain DNA Sequence

15 The manufacturer or user has a substantial choice in the range and variety  
16 of the DNA sequences suitable for use as the extracellular domain. A  
17 representative, but non-exhaustive, listing of suitable choices is provided by Table  
18 1 below.

19

20

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Table 1: Representative Extracellular Domain DNA Sequence Fragments

Extracellular Domain <u>Type Variant</u>	DNA Sequence <u>Recited By</u>
syndecan-1	Fig. 2
syndecan-2	Fig. 3
syndecan-3	Fig. 4
syndecan 4	Fig. 5
glypican-1	Fig. 6

## B. The Transmembrane Domain DNA Sequences

The manufacturer or user also has substantial choice in the range and variety of the DNA sequences to be used as the transmembrane domain sequence coding for the medial or central region of the expressed proteoglycan entity. A representative, but non-exhaustive, listing of the second DNA sequence in the prepared fragment constituting and coding for the transmembrane domain is provided by Table 2 below.

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Table 2: Representative Transmembrane Domain DNA Sequence Fragments

Transmembrane Domain <u>Type Variant</u>	DNA Sequence <u>Recited By</u>
syndecan-1	Fig. 7
syndecan-2	Fig. 8
syndecan-3	Fig. 9
syndecan 4	Fig. 10
GPI	Fig. 11
perlecan	Fig. 12



### C. The Cytoplasmic Domain Coding For The Syndecan-4 Peptide

The third requisite cytoplasmic domain must code for the amino acid residue structure representative of the syndecan-4 core protein. As shown experimentally by the data presented hereinafter, only the syndecan-4 cytoplasmic region and peptide structure allows for functional stimulation of angiogenesis in-situ. For this reason, it is essential and required in each embodiment of the present invention that the third DNA sequence coding for the cytoplasmic domain in the expressed proteoglycan entity in a transfected endothelial cell be representative of and analytically identifiable as the syndecan-4 amino acid residue structure. A representative recitation of the DNA constituting the cytoplasmic domain of the syndecan-4 molecule is presented by Fig. 13 herein.

It will be noted and recognized that very little variability and substitution within the specific DNA base sequencing of the cytoplasmic domain of the syndecan-4 molecule is permitted. While some changes are expected, be they point mutations, block substitutions and the like, the expected or envisioned degree of variability which might be present or permitted for the cytoplasmic domain DNA is believed to be quite limited.

As representative examples: The last four amino acids (EFYA) cannot be changed or modified. Similarly, regarding the Serine residue at position 181: a mutation to an Alanine residue potentiates activation; while a mutation to Glutamate inhibits cell growth in a dominant fashion (dominant-negative mutation). Finally, the LGKKPIYKK sequences probably cannot be altered at all.

## Expression Vectors And Means For Delivery In-Situ

A variety of methods are conventionally known and presently available to the user or practitioner of the present invention in order to introduce and deliver a prepared DNA sequence fragment to the intended target in-situ. The means for delivery envision and include in-vivo circumstances; ex-vivo specimens and conditions; and in-vitro culture circumstances. In addition, the present invention intends and expects that the use of the prepared DNA sequence fragment in a suitable expression vector and route of administration will be delivered to living tissues comprising endothelial cells, and typically vascular endothelial cells which constitute the basal layer of cells in capillaries and blood vessels generally. Clearly, the cells themselves are thus eukaryotic, typically mammalian cells from human and animal origin; and most typically would include the higher order mammals such as humans and domesticated animals kept as pets or sources of food intended for consumption. Accordingly, the range of animals includes all domesticated varieties involved in nutrition including cattle, sheep, pigs and the like; as well as those animals typically used as pets or raised for commercial purposes including horses, dogs, cats, and other living mammals typically living with and around humans.

Clearly, the expression vectors then must be suitable for transfection of endothelial cells in living tissues of mammalian origin and thus be compatible with that type and condition of cells under both in-vivo and/or in-vitro conditions. The expression vectors thus typically include plasmids and viruses as expression vectors.

1           The range and variety of plasmids suitable for use with the present  
2 invention are broadly available and conventionally known in the technical and  
3 scientific literature. A representative, but non-exhaustive, listing is provided by  
4 Table 3 below.

5

Table 3: Preferred Mammalian Plasmid Expression Vectors

1	
2	
3	
4	Plasmid Vectors
5	
6	pH $\beta$ -APr-1-neo
7	
8	EBO-pcD-XN
9	
10	pcDNAI/amp
11	
12	pcDNAI/neo
13	
14	pRc/CMV
15	
16	pSV2gpt
17	
18	pSV2neo
19	
20	pSV2-dhfr
21	
22	pTk2
23	
24	pRSV-neo
25	
26	pMSG
27	
28	pSVT7
29	
30	pKo-neo
31	
32	pHyg
33	
34	
35	
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37	

1           Alternatively, a wide and divergent variety of viral expression vectors  
2           suitable for insertion of the prepared DNA sequence fragment and subsequent  
3           transfection of endothelial cells in-situ is conventionally known and commonly  
4           available in this field. The particular choice of viral vector and the preparation of  
5           the fully constructed expression vector incorporating the prepared DNA sequence  
6           fragment is clearly a matter of personal convenience and choice to the intended  
7           manufacturer or user; but should be selected with a eye towards the intended  
8           application and the nature of the tissues which are the intended target. A  
9           representative, but non-exhaustive, listing of preferred viral expression vectors  
10          suitable for use as constructed vectors bearing the prepared DNA sequence  
11          fragment is provided by Table 4 below.

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Table 4: Preferred Viral Expression Vectors

- Bovine papilloma virus (BPV-1);
- Epstein-Barr virus (phEBO; pREP- derived, and p205);
- Retrovirus;
- Adenovirus;
- AAV (adeno-associated virus)
- Lentivirus

1           Clearly, both the plasmid based vectors and the viral expression vectors  
2           constitute means and methods of delivery which are conventionally recognized  
3           today as "gene therapy" modes of delivery. However, this overall approach is not  
4           the only means and method of delivery available for the present invention.

5  
6           Injection of recombinant proteins

7           Intracoronary delivery is accomplished using catheter-based deliveries of  
8           recombinant human protein dissolved in a suitable buffer (such as saline) which  
9           can be injected locally (i.e., by injecting into the myocardium through the vessel  
10          wall) in the coronary artery using a suitable local delivery catheter such as a 10mm  
11          InfusaSleeve catheter (Local Med, Palo Alto, CA) loaded over a 3.0mm x 20mm  
12          angioplasty balloon, delivered over a 0.014 inch angioplasty guidewire. Delivery  
13          was accomplished by first inflating the angioplasty balloon to 30 psi, and then  
14          delivering the protein through the local delivery catheter at 80 psi over 30  
15          seconds (this can be modified to suit the delivery catheter).

16          Intracoronary bolus infusion can be accomplished by a manual injection of  
17          the protein through an Ultrafuse-X dual lumen catheter (SciMed, Minneapolis,  
18          MN) or another suitable device into proximal orifices of coronary arteries over 10  
19          minutes.

20          Pericardial delivery is accomplished by instillation of the protein-containing  
21          solution into the pericardial sac. The pericardium is accessed either via a right  
22          atrial puncture, transthoracic puncture or via a direct surgical approach. Once the  
23          access is established, the material is infused into the pericardial cavity and the



1 catheter is withdrawn. Alternatively, the delivery is accomplished using slow-  
2 release polymers such as heparin-alginate or ethylene vinyl acetate (EVAc). In  
3 both cases, once the protein is integrated into the polymer, the desired amount of  
4 polymer is inserted under the epicardial fat or secured to the myocardial surface  
5 using, for example, sutures. In addition, polymer can be positioned along the  
6 adventitial surface of coronary vessels.

7 Intramyocardial delivery can be accomplished either under direct vision  
8 following thoracotomy or using thoracoscope or via a catheter. In either case, the  
9 protein containing solution is injected using a syringe or other suitable device  
10 directly into the myocardium. Up to 2 cc of volume can be injected into any given  
11 spot and multiple locations (up to 30 injections) can be done in each patient.  
12 Catheter-based injections are carried out under fluoroscopic, ultrasound or  
13 Biosense NOGA guidance. In all cases after catheter introduction into the left  
14 ventricle the desired area of the myocardium is injected using a catheter that allows  
15 for controlled local delivery of the material.

### 16 17 III. Exemplary Applications And Preferred Routes Of Administration

18 A variety of approaches, routes of administration, and delivery methods are  
19 available using the constructed expression vector comprising an inserted DNA  
20 sequence fragment coding for a proteoglycan entity. A majority of the approaches  
21 and routes of administration described hereinafter are medical applications and  
22 specific clinical approaches intended for use with human patients having specific  
23 medical problems and pathologies. It is expected that the reader is familiar

1 generally with the typical clinical human problem, pathology, and medical  
2 conditions described herein; and therefore will be able to follow and easily  
3 understand the nature of the intervention clinically using the present invention and  
4 the intended outcome and result of the clinical treatment - particularly as pertains  
5 to the stimulation of angiogenesis under in-vivo treatment conditions. A  
6 representative listing of preferred approaches is given by Table 5 below.

7

8

Table 5:

Preferred Routes Of Administration

Catheter-based (intracoronary) injections and infusions;

Direct myocardial injection

(intramyocardial guided);

Direct myocardial injection

(direct vision-epicardial-open chest or under thorascope guidance);

Local intravascular delivery;

Liposome-based delivery;

Delivery in association with "homing" peptides.

## Experimental and Empirical Data

To demonstrate the merits and value of the present invention, a series of planned experiments and empirical data are presented below. It will be expressly understood, however, that the experiments described and the results provided are merely the best evidence of the subject matter as a whole which is the invention; and that the empirical data, while limited in content, is only illustrative of the scope of the invention envisioned and claimed.

### A. Materials and Methods:

#### Expression constructs and cell culture

Immortalized ECV304 cells (ATCC, Bethesda, MD) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with heat-inactivated 10% fetal bovine serum (FBS, Gibco-BRL), 2 mM glutamine, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin at 37°C in 5% CO<sub>2</sub>. Full length coding region cDNAs for rat syndecan-4 and rat glypican-1 expression constructs were prepared in a retroviral vector MSCV2.2 by cloning a BamHI/HpaI fragment of rat syndecan-4 into cDNA into Bg<sup>L</sup>II/HpaI fragment vector and BamHI/EcoR<sup>L</sup>I fragment of rat glypican-1 into Bg<sup>L</sup>II/<sup>Eco</sup>R1 sites of the same vector.

Syndecan/glypican chimeras were created via PCR mutagenesis; cloned into the pCDNA3; sequenced; and shuttled into the MSCV2.2 vector. The syndecan-4-GPI (S4-GPI) construct was created by deleting the C-terminal end of rat syndecan-4

sequence starting with <sup>247</sup>Gln and replacing it with the C-terminal sequence of rat glypican-1 starting with <sup>510</sup>Ser. The glypican-syndecan-4 cytoplasmic domain (G1-S4c) construct was created by replacing C-terminal sequence of rat glypican-1 starting with <sup>510</sup>Ser with amino acids 247-321 of the rat syndecan-4 sequence. The created chimera thus contains both transmembrane and cytoplasmic regions of syndecan-4. Transfection of the MSCV2.2 vector alone was used to generate a control ECV cell population.

#### Retroviral transduction

The virus for transductions was produced by calcium phosphate transient transfection (29) of 10 µg of each construct on amphotropic Phoenix packaging cells (ATCC). Viral supernatants were collected after 36, 48 and 72 hrs, sterile filtered through 0.2 µm filter and then transferred to ECV-304 cells at 32°C in the presence of 25 µg/ml DEAE-dextran. Typical viral titers in the supernatant were approximately 6-8 x 10<sup>5</sup> infectious particles/ml. Virus exposure was repeated 4 times for each construct; following the last exposure the cells were cultured in 10% FBS-DMEM supplemented with 400 µg/ml active G418 (Sigma) for two weeks.

#### Growth and migration assays

For growth assays, 100,000 cells were plated in 6 well cell culture plates and allowed to attach overnight. At that time, the cells were washed 3 times with phosphate-buffered saline (PBS) and the medium was changed to DMEM

1 supplemented with 0.25% FBS. Twenty four hours later, 25 ng/ml of bFGF  
2 (Chiron Corp.) were added to the cell culture medium. Cell counts were then  
3 obtained at 24 hr intervals starting with the time of exposure to bFGF by  
4 trypsinizing the well and counting cell suspensions on a Coulter counter (Coulter  
5 Corp.).

6 Migration assays were carried out using modified Boyden chambers  
7 (Neuroprobe, Inc.). ECV 304 cells and derived clones were grown in 10% FBS-  
8 DMEM supplemented with 5 ng/ml DiI (DiI<sub>C18</sub>; 1,1-dioctadecyl-3,3,3',3'-  
9 tetramethylindocarbocyanide perchlorate, Molecular Probes) living cell fluorescent  
10 stain overnight. Following that, the cells were trypsinized, washed with DMEM,  
11 diluted in DMEM supplemented with 0.5% FBS and seeded in wells at 60,000  
12 cells per well. The cell containing compartments were separated from the lower  
13 wells by 25 x 80 mm polycarbonate filters with 8  $\mu$ m pores (Poretics Corp.). The  
14 lower chambers were filled with 0.5% FBS-DMEM supplemented with 50 ng/ml  
15 bFGF and the entire apparatus was incubated in a tissue culture incubator at 37°C,  
16 5% CO<sub>2</sub> for 4.5 hours. After that time non-migrating cells were removed by  
17 washing the upper wells with PBS, the upper surfaces of the filters were scraped  
18 with a plastic blade, and the filters were fixed in 4% formaldehyde for 1 min and  
19 placed on a glass slide. The migrated cells were imaged using a digital SesSys  
20 camera attached to a Nikon fluorescent microscope. For each slide, 3 non-  
21 overlapping lower power (5x) fields were selected for analysis. Following image  
22 acquisition using PMIS image processing software (Photometrics, Ltd.) the number  
23 of cells was automatically determined using Optimas 6.0 software (Bioscan, Inc.).

### Matrigel growth assay

Growth factor depleted Matrigel (Becton Dickinson) plates were prepared by adding 0.5 ml of thawed Matrigel to a well of refrigerated 24 well tissue culture plate. The gel was allowed to solidify for one hour at 37°C and overlaid with 1 ml of 0.5% FBS-DMEM containing 30,000 cells. The cell culture was carried out at 37°C in a humidified atmosphere supplemented with 5% CO<sub>2</sub>. The analysis of cell growth was carried out by obtaining lower (10x) and high (40x) power images of the wells with a digital SesSys camera focused on the surface of the gel using an inverted Nikon fluorescent microscope. The cell-free area was the determined using Optimas 6.0 software.

### RNA Isolation and RT PCR Analysis

For RNA analysis of syndecan-4 and PR-39 expression, cell cultures were trypsinized, pelleted, and total RNA was prepared using TRI Reagent (Sigma Biosciences). The RNA pellet was dissolved in RNase-free water and ethanol precipitated. For RT-PCR analysis, 0.2 µg total RNA were used for reverse transcription with a 15 pmol of oligo(dT)<sub>20</sub> primer, 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM DTT, 0.5 mM each dNTP in 50 mM Tris-HCl (pH 8.3) buffer. The mixture was heated to 70°C for 10 min, then cooled to 37°C while 1 µl of Super Script II reverse transcriptase (200 U/µl, Life Technologies, Inc.) was added. The reaction was allowed to proceed for 1 hr at 37°C and then terminated by heating for 5 min followed by chilling to 4°C. 1 µl of the RT reaction mixture was used for PCR amplification using specific primers. The PCR reaction was carried out

1 in the presence of 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 400 nM 3' and 5' primers and  
2 2.5 U of Taq DNA polymerase (Boehringer Mannheim, Inc.). The following  
3 specific primers were used: Glypican-1: 5': CCC CGC CAG CAA GAG CCG  
4 GAG CT; 3': GTG AGG CTC TGG GCG AGT GGG GG, Syndecan-4: 5' (with  
5 Sac I restriction site): ATA GAG CTC TTG GAA CCA TGG CFC CTG TCT  
6 GCC; 3': (with Eco RI restriction site): GGA ATT CCA GGT TTT ATT ATC  
7 TTT TTA TC.

8 For standardization purposes a conserved region of human and mouse GAP-  
9 DH gene was chosen for amplification as a control template. The following  
10 primers were used: 5': CGT ATT GGG CGC CGT GTC ACC AGG GC; 3':  
11 GGC CAT GAG CTC CAC CAC CCT GTT CG. All <sup>PCR</sup> reactions were  
12 carried out using GeneAmp PCR 2400 system (Perkins Elmer, Inc.) as follows:  
13 94°C (1 min), 50-55°C (30 sec), 72°C (1.5 min). The additional final extension  
14 step was performed at 72°C for 7 min. A total of 30 cycles were done for each  
15 reaction. Following PCR amplification, reaction products were subjected to 1 %  
16 agarose gel electrophoresis and the amount of specific message was expressed as a  
17 ratio to GAP-DH message.

#### 18 Determination of heparan sulfate mass in cultured cells

19 To determine the total mass of heparan sulfate chains, endothelial cell  
20 cultures were washed twice with PBS and incubated for 24 h with 2 mCi of  
21 Na<sub>2</sub><sup>35</sup>SO<sub>4</sub> in 2 ml of a modified basal Eagle medium supplemented with 1 %  
22 Neutrodoma-SP. At the end of labeling, cells are washed with cold PBS and  
23



1 incubated with a lysis buffer followed by centrifugation at 15,000 x g for 10 min  
2 at 4°C. Total proteoglycans (PG) are isolated from the supernatant by DEAE  
3 chromatography. Glycosaminoglycans were cleaved from the total PG pool by  
4  $\beta$ -elimination and the relative content of HS and CS is determined by appropriate  
5 enzyme digests with chondroitinase ABC or *Flavobacterium* heparatinase 1 and 3.  
6 Preliminary experiments on microvascular endothelial cells demonstrated that the  
7 sum of HS and CS sulfate accounted for >98% of the total PG sulfate.

#### 8 9 Scatchard analysis of low affinity bFGF binding sites

10 For determination of the number and affinity of bFGF heparan sulfate  
11 binding sites, endothelial cells were grown to near confluence in 24 well dishes in  
12 10% FBS-DMEM. After two washes with cold PBS, 200  $\mu$ l of binding buffer  
13 (25 mM HEPES, pH 7.4, 0.1% BSA, 0.05% gelatin in M199 medium),  $6 \times 10^6$   
14 cpm (0.5 ng/ml)  $^{125}$ -I-bFGF (DuPont, specific activity 2000 C/mmol), and  
15 increasing amounts (0-600 ng/ml) cold bFGF were added to each well. The cells  
16 were incubated at 4°C for 2 h with gentle agitation; at the end of that time, the  
17 cells were washed three times with 1 ml PBS containing 0.1% BSA and then  
18 incubated with 1% Triton-X 100 in 5 ml water supplemented with 0.01% BSA  
19 (Sigma) for 30 min at room temperature with vigorous shaking. Following this,  
20 0.4 ml aliquots were counted in a 1272 CliniGamma counter (LKB). Cell counts  
21 determined by a Coulter Counter were employed to establish the number of cells  
22 per well. Background counts were subtracted from all samples. Scatchard  
23 analysis of the specifically bound material vs. the molar amount of cold competitor

was carried out using Origin 5.0 software (Microcal Software, Inc., Northampton, MA). All experiments were carried in triplicate and repeated at least twice.

## B. Empirical Data and Results

### Experimental Series I

This series of experiments is directed to demonstrating the role of cell associated heparan sulfate chimeric core proteins in endothelial cells in-situ. The bulk of the experiments and empirical data in this series are in-vitro results.

#### Experiment 1:

The immortalized human endothelial cell line ECV304 was transfected with prepared retroviral constructs containing full length cDNAs for either syndecan-4 or glypican-1. In addition, in order to differentiate potential biological effects secondary to increased mass of cell surface and/or extracellular heparan sulfates versus increased core protein expression, two additional chimera core protein constructs were created. In one, S4-GPI, syndecan 4 extracellular domain was linked to the glypican 1 GPI anchored; and in another, G1-S4c, the extracellular domain of glypican 1 was linked to the transmembrane and cytoplasmic domains of syndecan-4. Cells transfected with a vector only construct (ECV-VC) were used as control. Increased expression of both syndecan-4 and glypican-1 constructs was

expected to result in larger numbers of heparan sulfate chains on the cell surface.

Subsequently, the total mass of heparan sulfate chains on the wild type as well as the 4 newly generated transfected ECV cell lines was determined. Total heparan sulfate mass was significantly increased (per  $\mu\text{g}$  of total cellular protein) in ECV-S4, ECV-G1, ECV-S4-GPI and ECV-G1-S4c but not ECV-VC cells. This data is presented by Table E1.

In order to assess whether these changes in HS expression resulted in selective alterations of heparan binding growth factors, the low affinity binding of bFGF, a prototypical heparin binding growth factor was examined. Scatchard analysis of the wild type and newly generated transfected ECV cell lines showed that there were no significant changes in the affinity of bFGF binding (see Table E2; mean of 3 experiments). At the same time, there was a 2-fold increase in the number of bFGF binding sites in S4 and C1-S4c clones and somewhat smaller increase in ECV-G1 and ECV-S4-GPI clones (Table E2). The smaller increase in cell-associated HS mass in glypican and syndecan-4 GPI overexpressers was expected given higher shedding rates for GPI-linked glypican compared to the transmembrane syndecan. Also, the increase in the number of bFGF binding sites was of the same order as the increase in the total HS cell mass -- thus showing that there was no preferential creation of bFGF binding sites and, there was no significant change in the bFGF-HS/HS ratio (calculated as ratio of a relative increase in the number of HS-bFGF sites per cell and a relative increase in the total HS mass). Thus, for a ECV-S4 clone compared to control, there was a  $5.94 \times 10^6 / 2.32 \times 10^6 = 2.56$  fold increase in the number of bFGF-HS sites (Table E2)

1 and a  $0.33/0.14=2.36$  increase in the total HS mass (per  $\mu\text{g}$  protein, Table E1)  
2 giving the HS-bFGF/HS ratio of  $2.36/2.56=0.75$ .  
3  
4

Table E1: HS Mass In Various Stable Clones

	<u><sup>35</sup>S HS / g protein</u>
ECV-VC	0.14±0.026
ECV-S4	0.33±0.042
ECV-G1	0.23±0.015
ECV-S4-GPI	0.24±0.080
ECV-G1-S4c	0.34±0.050

<sup>35</sup>S counts in HS expressed per g of total protein.

Table E2: Effect of S4, G1 and chimera constructs expression on low affinity Kd and the number of binding sites for bFGF

	<u>Kd</u>	<u>Number of sites per cell</u>	<u>HS-bFGF / Total HS Ratio</u>
ECV-VC	0.60 * 10 <sup>-9</sup>	2.32 * 10 <sup>6</sup>	1.00
ECV-S4	0.85 * 10 <sup>-9</sup>	5.94 * 10 <sup>6</sup>	0.92
ECV-G1	0.81 * 10 <sup>-9</sup>	3.60 * 10 <sup>6</sup>	0.95
ECV-S4-GPI	0.69 * 10 <sup>-9</sup>	3.80 * 10 <sup>6</sup>	0.96
ECV-G1-S4c	0.53 * 10 <sup>-9</sup>	4.89 * 10 <sup>6</sup>	0.87

Experiment 2:

To study the effect of syndecan-4 and glypican-1 expression on endothelial cell growth, the ability of wild type and newly created ECV cell lines to grow in vitro in response to serum and bFGF was analyzed. Experimentally, all cells were growth arrested for 48 hours and then stimulated with <sup>0.25%</sup> FBS supplemented with <sup>25</sup> ng/ml bFGF. The data is shown by Fig. 14 in which, MSCV-ECV-vector control; G1: glypican-1 full length cDNA; S4-GPI; syndecan-4 extracellular domain linked to the GPI anchor; S4: full length syndecan-4 cDNA; G1-S4c: extracellular domain of glypican-1 linked to syndecan-4 transmembrane/ cytoplasmic domain.

As shown therein, the ECV-S4 and ECV-G1-S4c cells demonstrated a 4-fold increase in cell number compared to ECV wild type or vector-transfected (MSCV) cells. At the same time, growth of ECV-G1 or ECV-S4-GPI cells did not differ significantly from wild type ECV cells. Even though both ECV-G1 and ECV-S4-GPI clones had somewhat smaller numbers of bFGF-HS binding sites per cell, the absence of any significant change in bFGF growth response is out of proportion to the magnitude of HS-bFGF increase.

Experiment 3:

To test the effect of these constructs expression on the cells ability to form vascular structures, wild type and newly generated ECV clones were plated on Matrigel in 10% FBS-DMEM. Three days later, the presence of definable structures (cords and rings) was assayed by light microscopy. As in the case of in-

1 vitro growth assays, ECV-S4 and ECV-G1-S4c cells formed more numerous and  
2 denser vascular structures compared to wild type ECV, ECV-G1 or ECV-S4-GPI  
3 cells. The results are shown by Figs. 15A-15C.

4 As seen in Figs. 15A-15C respectively, vector transduced ECV cells  
5 (MSCV) as well as ECV transduced with full length syndecan-4 and G1-S4c  
6 construct-carrying retroviruses were plated on growth factor depleted Matrigel  
7 supplemented with 25 ng/ml bFGF. Photographs of the gels were taken 72 hours  
8 later. Note the presence of increased vascular networks and cell density in S4 and  
9 G1-S4c panels compared to MSCV panel.

#### 10 11 Experiment 4:

12 To further analyze the effect syndecan, glypican, or syndecan/glypican  
13 chimeras expression on biological behavior of endothelial cells, the migration of  
14 wild type and generated ECV cell lines migration towards serum and bFGF in  
15 Boyden chamber assays was analyzed. Similar to the growth assay results, the cell  
16 lines expressing increased amounts of syndecan-4 or glypican-syndican-4  
17 cytoplasmic tail chimeras demonstrated a significantly higher ability to migrate  
18 compared to wild type ECV or ECV expressing glypican-1 or extracellular domain  
19 of syndecan-4 linked to the glypican-1 GPI anchor. This is shown by Fig. 16.

#### 20 21 Overall Conclusions:

22 The experiments demonstrate, therefore, that syndecan-4 expression  
23 resulted in significant increase in bFGF-stimulated growth of EC in 2-D and 3-D

1 cultures as well as in enhanced migration towards the bFGF gradient. These  
2 results cannot be attributed to the increase in HS cell mass or preferential creation  
3 of low affinity (HS) bFGF binding sites rather than increased syndecan-4 core  
4 protein expression, since overexpression of glypican-1 while producing the same  
5 increase in HS mass did not produce increased growth and migration responses to  
6 bFGF. This conclusion is further supported by observation that while glypican-S4  
7 cytoplasmic domain chimera closely mimicked effects of syndecan-4  
8 overexpression, syndecan-4-GPI chimera had no effect on bFGF responses in these  
9 cells. Finally, while both syndecan-4 and glypican1 expression increased total HS  
10 cell mass there was no significant change in the number of low or high (data not  
11 shown) affinity HS bFGF binding sites. Thus, increased expression of syndecan-4  
12 cytoplasmic domain is associated with increased responsiveness to bFGF  
13 stimulation as defined by cell growth and migration assays.

## 14 15 Experimental Series II

16  
17 The second experimental series is directed to demonstrating the role of  
18 climeric cone proteins in stimulating angiogenesis under in-vivo conditions. The  
19 experiments and data presented hereinafter are representative of clinical conditions  
20 and medical pathologies in living humans and animals.  
21  
22



1 Experiment 5:

2 To demonstrate the role and effect of chimeric cone protein in regulation of  
3 angiogenesis in-vivo, a rat myocardial infarction model [as reported in Li et al.,  
4 Am. J. Physiol. 270: H1803-H1811 (1997)] was adapted to in-vivo studies using  
5 mice.

6 In this model, ligation of a proximal coronary artery leads to reproducible  
7 infarction accompanied by peri-infarction angiogenesis that can be characterized in  
8 a number of ways including in-situ hybridization, immunocytochemistry and  
9 morphometric analysis. Using this model, rapid (within 1 hour) induction of  
10 syndecan-4 gene expression in peri-infarct region that was dependent on the influx  
11 of blood-derived macrophages was demonstrated. A comparison of the extent of  
12 angiogenesis in macrophage-deficient homozygous op/op mice (low post-MI  
13 syndecan expression) to that in the op/op mice treated with GM-CSF (thus  
14 restoring macrophage population and syndecan-1/4 expression) revealed a 4 fold  
15 increase in neovascularization in the latter as determined by BudR intake and  
16 morphometric analysis. This result is shown by Figs. 17A-17F respectively.

17 Figs. 17A-17F show BudR uptake in op/op homozygous (-/-) and  
18 heterozygous (+/-) mice over 3 days time. Note the intense BudR uptake by cells  
19 on the infarct periphery in (+/-) mice but not in (-/-) mice within the per-infarct  
20 area on both day 1 and day 3 post-infarction.

Experiment 6:

To further link syndecan expression to enhanced angiogenic response in these settings, transgenic mice lines were generated with cardiac myocyte-specific expression of PR-39 peptide using  $\alpha$ -MHC promoter. The PR-39 peptide has been shown to increase both syndecan-1 and syndecan-4 expression in-vitro in a variety of cell types. [See for example, Gallo *et al.*, Proc. Natl. Acad. Sci. USA **91**: 11035-11039 (1994) and Li *et al.*, Circ. Res. **81**: 785-796 (1997)].

Analysis of syndecan gene expression in PR-39 transgenic mice demonstrated marked increase in expression of syndecan-4 and glypican-1 genes. This is shown by Fig. 18. Equally important, there was no detectable expression of syndecan-1 in either wild type or transgenic mice (data not shown).

Immunocytochemical analysis with anti-CD31<sup>antibody</sup> demonstrated increased vascular density in PR-39 transgenics and the morphometric analysis confirmed a 3 fold increase in the number of capillaries and small (<200  $\mu$ m diameter) diameter vessels in these mice.

In particular, Fig. 18 shows a Northern blot analysis of gene expression in PR-39 transgenic mice. The LV myocardium from the wild type (WT) and two PR-39 transgenic lines (A,B) mice was subjected to Northern blot analysis. Note the increased syndecan-4 and glypican-1 expression in both transgenic mice compared to WT mice.

Experiment 6:

To confirm the functional significance of this increase in vascularity, the total coronary resistance was assessed in an isolated heart preparation as previously described [Li et al., *J. Clin. Invest.* 100: 18-24 (1997)]. In these settings, a 2 fold decrease in coronary perfusion pressure was observed for any given perfusion rate, thus confirming a reduced transmyocardial resistance to flow. To further evaluate vascular function in these mice, a study of bFGF-induced vasodilation in microvascular preparations in-vitro demonstrated an increased bFGF sensitivity of PR-39 mice vessels. This is shown by the data of Fig. 19.

As presented, Fig. 19 provides an in-vitro assessment of microvascular reactivity. Microvascular preparations from PR-39 transgenic and control mice were precontracted with endothelin and then evaluated for a vasodilatory response to an endothelium-dependent agents ADP and bFGF. Note that while both PR-39 transgenics and controls are equally responsive to ADP, bFGF response is much more profound in the PR-39 mice (\*  $p < 0.05$ ).

Overall Conclusions:

Myocardial-specific expression of PR-39 resulted in increased expression of syndecan-4 and glypican-1 genes that was accompanied by a functionally significant increase in coronary vascularity and enhanced bFGF responsiveness. These studies, therefore, provide rational evidence and direct support for the in-vivo efficacy of climeric cone protein expression in angiogenic stimulation.

1           The present invention is not to be limited in scope nor restricted in form  
2 except by the claims appended hereto.  
3  
4

What we claim is:

1. A prepared DNA segment for placement in a suitable expression vector and transfection of endothelial cells in-situ such that overexpression of extracellular matrix heparan sulfate proteoglycan entities subsequently occurs in-situ, said prepared DNA segment comprising:

at least one first DNA sequence coding for the extracellular domain of a discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ, said extracellular domain first DNA sequence specifying the extracellular N-terminal portion of an expressed proteoglycan entity which is then located at and extends from the endothelial cell surface and is capable of binding heparan sulfates to form an extracellular matrix in-situ;

at least one second DNA sequence coding for the transmembrane domain of a discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ, said transmembrane domain second DNA sequence specifying the medial portion of an expressed proteoglycan entity which is then located at and extends through the endothelial cell membrane and is joined with said extracellular N-terminal portion of said expressed proteoglycan entity; and

at least one third DNA sequence coding for the cytoplasmic domain of the syndecan-4 molecule in said discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ, said syndecan-4 cytoplasmic domain third DNA sequence specifying the cytoplasmic portion of an expressed proteoglycan entity which is then present within the cytoplasm of a transfected endothelial cell and is

joined to said transmembrane portion and said extracellular N-terminal portion of said expressed proteoglycan entity.

2. A constructed expression vector for transfection of endothelial cells in-situ such that overexpression of extracellular matrix haparan sulfate proteoglycan entities subsequently occurs in-situ, said constructed expression vector comprising:

a prepared DNA segment comprised of

(i) at least one first DNA sequence coding for the extracellular domain of a discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ, said extracellular domain first DNA sequence specifying the extracellular N-terminal portion of an expressed proteoglycan entity which is then located at and extends from the endothelial cell surface and is capable of binding heparan sulfates to form an extracellular matrix in-situ,

(ii) at least one second DNA sequence coding for the transmembrane domain of a discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ, said transmembrane domain second DNA sequence specifying the medial portion of an expressed proteoglycan entity which is then located at and extends through the endothelial cell membrane and is joined with said extracellular N-terminal portion of said expressed proteoglycan entity, and

(iii) at least one third DNA sequence coding for the cytoplasmic domain of the syndecan-4 molecule in said discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ, said syndecan-4 cytoplasmic

domain third DNA sequence specifying the cytoplasmic portion of an expressed proteoglycan entity which is then present within the cytoplasm of a transfected endothelial cell and is joined to said transmembrane portion and said extracellular N-terminal portion of said expressed proteoglycan entity; and

an expression vector carrying said prepared DNA segment and suitable for transfection of endothelial cells in-situ.

3. An in-situ transfected endothelial cell which overexpresses extracellular matrix heparan sulfate proteoglycans and positions on the proteoglycan entities at the cell surface, said in-situ transfected endothelial cell comprising:

✓ a viable endothelial cell previously transfected in-situ with a constructed expression vector such that said transfected endothelial cell overexpresses discrete extracellular matrix heparan sulfate proteoglycan entities coded for by said vector, said overexpressed proteoglycan entities being comprised of

(i) an extracellular N-terminal portion which is located at and extends from the transfected endothelial cell surface and which binds heparan sulfates to form an extracellular matrix in-situ, said extracellular N-terminal portion being the expressed product of at least one first DNA sequence in the prepared expression vector coding for the extracellular domain of said proteoglycan entity expressed by the transfected endothelial cell in-situ,

(ii) a transmembrane medial portion which is located at and extends through the endothelial cell membrane and is joined with said extracellular N-terminal portion of said proteoglycan entity, said transmembrane medial portion

being the expressed product of at least one second DNA sequence in the prepared expression vector coding for the transmembrane domain of said proteoglycan entity expressed by the transfected endothelial cell in-situ, and

(iii) a syndecan-4 cytoplasmic portion present within the cytoplasm of the transfected endothelial cell which is joined to said transmembrane portion and said extracellular N-terminal portion of said proteoglycan entity, said syndecan-4 cytoplasmic portion being the expressed product of at least one third DNA sequence in the prepared expression vector coding for the cytoplasmic domain of the syndecan-4 molecule of said proteoglycan entity expressed by the transfected endothelial cell in-situ.

4. The prepared DNA segment as recited by claim 1 wherein said first DNA sequence coding for the extracellular domain of a discrete proteoglycan entity is selected from the group consisting of syndecan DNA sequences, glypican DNA sequences and perlecan DNA sequences.

5. The prepared DNA segment as recited by claim 1 wherein said second DNA sequence coding for the transmembrane domain of a discrete proteoglycan entity is selected from the group consisting of syndecan DNA sequences, glypican DNA sequences and perlecan DNA sequences.

6. The constructed expression vector as recited by claim 2 wherein said expression vector suitable for transfection of endothelial cells in-situ is a plasmid.



7. The constructed expression vector as recited by claim 2 wherein said expression vector suitable for transfection of endothelial cells in-situ is a virus.

8. The in-situ transfected endothelial cell as recited by claim 3 wherein said cell is selected from the group consisting of vascular endothelial cells and dermal endothelial cells.

9. The in-situ transfected endothelial cell as recited by claim 3 wherein said cell exists under in-vivo conditions.

10. The in-situ transfected endothelial cell as recited by claim 3 wherein said cell exists under in-vitro conditions.

11. The in-situ transfected endothelial cell as recited by claim 3 wherein said transfected endothelial cell exists in a tissue comprising at least one kind of muscle cell selected from the group consisting of myocardial muscle cells, smooth muscle cells and striated muscle cells.

12. A method for making a prepared DNA segment intended for placement in a suitable expression vector and transfection of endothelial cells in-situ such that overexpression of extracellular matrix heparan sulfate proteoglycan entities subsequently occurs in-situ, said method comprising the steps of:

obtaining at least one first DNA sequence coding for the extracellular domain of a discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ, said extracellular domain first DNA sequence specifying the extracellular N-terminal portion of an expressed proteoglycan entity which is then located at and extends from the transfected endothelial cell surface and is capable of binding heparan sulfates to form an extracellular matrix in-situ;

acquiring at least one second DNA sequence coding for the transmembrane domain of a discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ, said transmembrane domain second DNA sequence specifying the medial portion of an expressed proteoglycan entity which is then located at and extends through the transfected endothelial cell membrane and is joined with said extracellular N-terminal portion of said expressed proteoglycan entity;

procuring at least one third DNA sequence coding for the cytoplasmic domain of the syndecan-4 molecule in said discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ, said syndecan-4 cytoplasmic domain third DNA sequence specifying the cytoplasmic portion of an expressed proteoglycan entity which is then present within the cytoplasm of a transfected endothelial cell and is joined to said transmembrane portion and said extracellular N-terminal portion of said expressed proteoglycan entity; and

joining together said extracellular domain first DNA sequence, said transmembrane domain second DNA sequence, and said syndecan-4 cytoplasmic domain third DNA sequence as a discrete prepared DNA segment.

13. A method for making a constructed expression vector intended for transfection of endothelial cells in-situ such that overexpression of extracellular matrix haparan sulfate proteoglycans subsequently occurs in-situ, said method comprising the step of:

obtaining a prepared DNA segment comprised of

(i) at least one first DNA sequence coding for the extracellular domain of a discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ, said extracellular domain first DNA sequence specifying the extracellular N-terminal portion of an expressed proteoglycan entity which is then located at and extends from the transfected endothelial cell surface and is capable of binding heparan sulfates to form an extracellular matrix in-situ,

(ii) at least one second DNA sequence coding for the transmembrane domain of a discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ, said transmembrane domain second DNA sequence specifying the medial portion of an expressed proteoglycan entity which is then located at and extends through the transfected endothelial cell membrane and is joined with said extracellular N-terminal portion of said expressed proteoglycan entity, and

(iii) at least one third DNA sequence coding for the cytoplasmic domain of the syndecan-4 molecule in a discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ, said syndecan-4 cytoplasmic domain third DNA sequence specifying the cytoplasmic portion of an expressed proteoglycan entity which is then present within the cytoplasm of a transfected

endothelial cell and is joined to said transmembrane portion and said extracellular N-terminal portion of said expressed proteoglycan entity; and

positioning said prepared DNA segment in an expression vector suitable for transfection of endothelial cells in-situ.

14. A method for stimulating angiogenesis in-situ within a living tissue comprising vascular endothelial cells, said method comprising the steps of:

transfecting vascular endothelial cells within a living tissue with a constructed expression vector such that the resulting transfected endothelial vascular cells overexpress discrete extracellular matrix heparan sulfate proteoglycan entities coded for by said constructed expression vector, said overexpressed proteoglycan entities being comprised of

(i) an extracellular N-terminal portion which is located at and extends from the transfected vascular endothelial cell surface and binds heparan sulfates to form an extracellular matrix in-situ, said extracellular N-terminal portion being the expressed product of at least one first DNA sequence in the constructed expression vector coding for the extracellular domain of said proteoglycan entity expressed by a transfected endothelial cell in-situ,

(ii) a transmembrane medial portion which is located at and extends through a transfected vascular endothelial cell membrane and is joined with said extracellular N-terminal portion of expressed proteoglycan entity, said transmembrane medial portion being the expressed product of at least one second DNA sequence in the constructed expression vector coding for the transmembrane

domain of said proteoglycan entity expressed by a transfected endothelial cell in-situ, and

(iii) a syndecan-4 cytoplasmic portion present within the cytoplasm of a transfected endothelial cell which is joined to said transmembrane portion and said extracellular N-terminal portion of said proteoglycan entity, said syndecan-4 cytoplasmic portion being the expressed product of at least one third DNA sequence in the constructed expression vector coding for the cytoplasmic domain of the syndecan-4 molecule of said proteoglycan entity expressed by a transfected endothelial cell in-situ; and

allowing said transfected vascular endothelial cells bearing said overexpressed extracellular matrix proteoglycan entities to stimulate angiogenesis in-situ.

15. The method for stimulating angiogenesis in-situ as recited by claim 14 wherein said living tissue comprises at least one other type of cell selected from the group consisting of muscle cells, fibrocytes and fibroblasts, epithelial cells, osteocysts and osteoblasts, erythrocytes and leukocytes, and neurons.

16. The method for stimulating angiogenesis in-situ as recited by claim 14 wherein said living tissue comprises at least one tissue selected from the group consisting of myocardium, lung, brain, kidney, spleen, liver, and gastro-intestinal tissues.

17. The method for stimulating angiogenesis in-situ as recited by claim 14 wherein said living tissue comprising vascular endothelial cells is transfected using means selected from the group consisting of catheter-based administration, injection-based administration, infusion-based administration, localized intravascular deliveries, liposome-based deliveries, and administrations using target-directed peptides.

18. The method for stimulating angiogenesis in-situ as recited by claim 14 wherein said method is practiced under in-vivo conditions.

19. The method for stimulating angiogenesis in-situ as recited by claim 14 wherein said method is practiced under in-vitro conditions.

## ABSTRACT OF THE INVENTION

The present invention provides tangible means and methods for stimulation of angiogenesis via enhanced endothelial expression of core proteins having a syndecan-4 cytoplasmic region intracellularly. The tangible means include a prepared DNA sequence fragment having separate and individual DNA sequenced portions coding for an heparan sulfate binding extracellular domain, a central transmembrane domain, and a cytoplasmic domain coding for the syndecan-4 polypeptide. The prepared DNA sequence unitary fragment may be delivered to endothelial cells in-situ, both under in-vivo and/or in-vitro conditions, using suitable expression vectors including plasmids and viruses. The resulting transfected endothelial cells overexpress heparan sulfate binding, core proteins; and the resulting overexpression of these proteoglycan entities causes stimulation of angiogenesis in-situ.

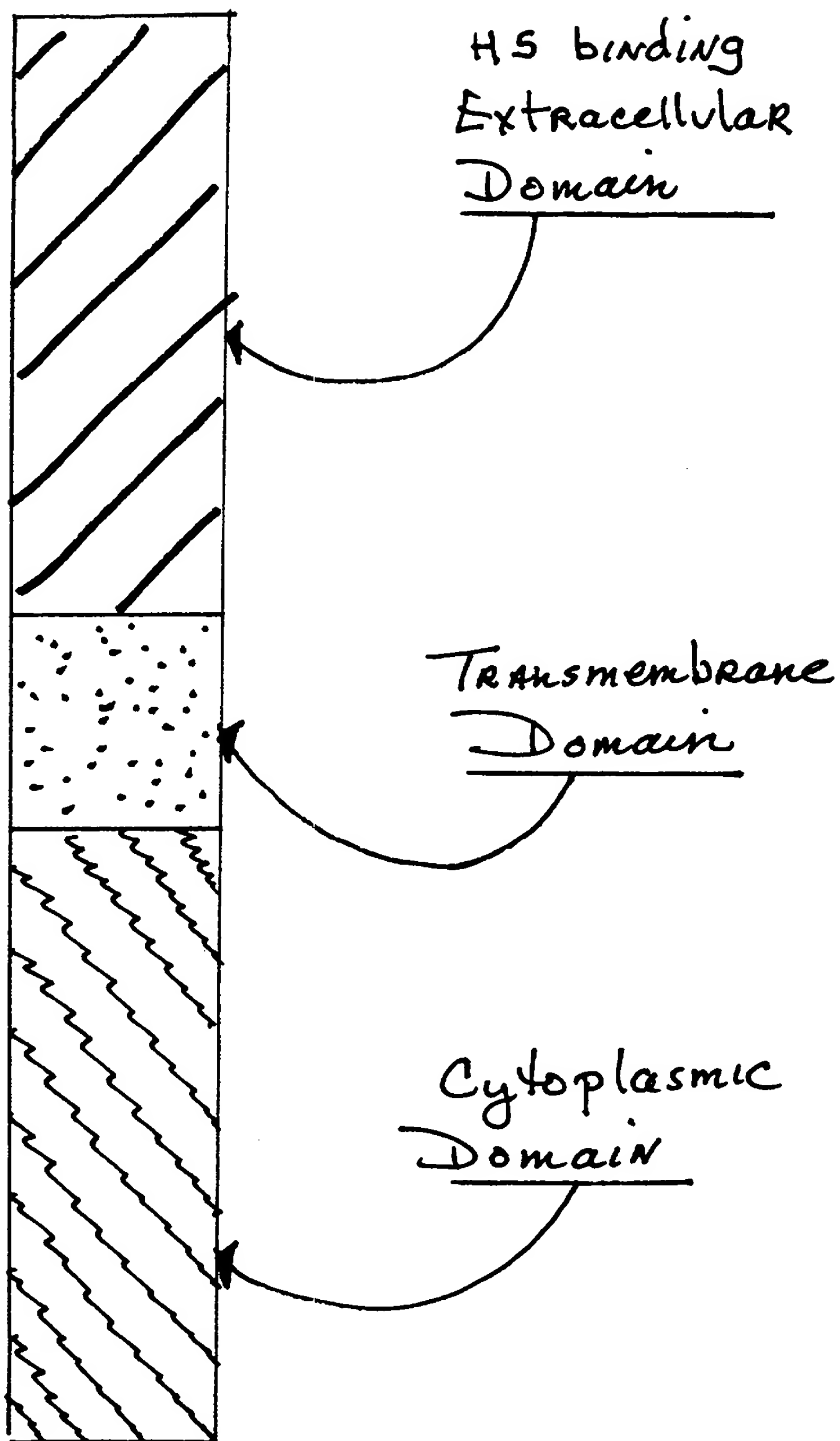


Fig. 1



**SECRET**

ACCESSION M81785  
LOCUS RATSYNDECA 2396 bp mRNA ROD 16-JUL-1992  
DEFINITION Rattus norvegicus syndecan mRNA, complete cds.  
ACCESSION M81785  
NID g207140  
KEYWORDS syndecan.  
SOURCE Rattus norvegicus Epididymal fat pad cDNA to mRNA.  
ORGANISM Rattus norvegicus  
Eukaryotae; mitochondrial eukaryotes; Metazoa; Chordata;  
Vertebrata; Eutheria; Rodentia; Sciurognathi; Myomorpha; Muridae;  
Murinae; Rattus.  
REFERENCE 1 (bases 1 to 2396)  
AUTHORS Kojima,T., Shworak,N.W. and Rosenberg,R.D.  
TITLE Molecular cloning and expression of two distinct cDNA-encoding  
heparan sulfate proteoglycan core proteins from a rat endothelial  
cell line  
JOURNAL J. Biol. Chem. 267, 4870-4877 (1992)

Fig. 3: Syndecan-2 (human) extracellular domain (J 04621):

ggcaggagggg agggagccag aggaaaagaa gaggaggaga aggaggagga cccggggagg  
gagggcgcggc gcgggaggag gaggggcgca gccgcggagc cagtggcccc gcttggacgc  
gctgctctcc agataccccc ggagctccag ccgcgcggat cgcgcgctcc cgccgctctg  
cccctaaact tctgccgtag ctccctttca agccagcgaa tttattcctt aaaaccagaa  
actgaacctc ggcacgggaa aggagtccgc ggaggagcaa aaccacagca gagcaagaag  
agcttcagag agcagccttc ccggagcacc aactccgtgt cgggagtgca gaaaccaaca  
agtgagaggg cgccgcgttc ccggggcgca gctgcgggag gcgggagcag gcgcaggagg  
aggaagcgag cgcccccgag ccccgagccc gagtccccga gcctgagccg caatcgctgc  
ggtactctgc tccggattcg tgtgcgcggg ctgcgcgagc gctggggcagg aggcttcgtt  
ttgccctggg tgcaagcagc ggctgggagc agccgggtccc tggggaatat gcggcgcgcg  
tggatcctgc tcaccttggg cttgggtggc tgcgtgtcgg cggagtcgag agcagagctg  
acatctgata aagacatgta ccttgacaac agctccattg aagaagcttc aggagtgtat  
cctattgatg acgatgacta cgcttctgcg tctggctcgg gagctgatga ggatgtagag  
agtccagagc tgacaacaac tcgaccactt ccaaagatac tgttgactag tgctgctcca  
aaagtggaaa ccacgacgct gaatatacag aacaagatac ctgctcagac aaagtcacct  
gaagaaactg ataaagagaa agttcacctc tctgactcag aaaggaaaat ggaccagcc  
gaagaggata caaatgtgta tactgagaaa cactcagaca gtctgtttaa acggacagaa

protein sequence:

GRREGARGKEEEEKEEDPGREARRGRRRGAAAEFVAPLGRAALQ  
IPPELQPRGSRAPAALPLNFCRSSLSSQRIYSLKPETEPRHGKGVRGGAKPQOSKKSF  
REQPSRSTNSVSGVQKPTSERAPRSRGAAAGGSRRRRRKRAPPSPEPESPSLSRNC  
GTLRLRVRGLAERWAGGFVLPWLQAAAGSSRLGNMRRRAWILLTLGLVACVSAESRA  
ELTSDKDMYLDNSSIEEASGVYPIDDDYASASGSGADEDVESPELTTRPLPKILLT  
SAAPKVETTLNIQNKIPAQTKSPEETDKEKVHLSDSERKMDPAEEDTNVYTEKHS  
LFRTE

reference:

ACCESSION J04621  
LOCUS HUMHSPGC 3414 bp mRNA PRI 08-NOV-1994  
DEFINITION Human heparan sulfate proteoglycan (HSPG) core protein, 3' end.  
ACCESSION J04621  
NID g184428  
KEYWORDS core protein; heparan sulfate proteoglycan.  
SOURCE Human fetal lung fibroblast, cDNA to mRNA, clone 48K5.  
ORGANISM Homo sapiens  
Eukaryotae; mitochondrial eukaryotes; Metazoa; Chordata;  
Vertebrata; Eutheria; Primates; Catarrhini; Hominidae; Homo.  
REFERENCE 1 (bases 1 to 3414)  
AUTHORS Marynen,P., Zhang,J., Cassiman,J.J., Van den Berghe,H. and David,G.  
TITLE Partial primary structure of the 48- and 90-kilodalton core  
proteins of cell surface-associated heparan sulfate proteoglycans  
of lung fibroblasts. Prediction of an integral membrane domain and  
evidence for multiple distinct core proteins at the cell surface of  
human lung fibroblasts  
JOURNAL J. Biol. Chem. 264 (12), 7017-7024 (1989)

## Fig. 4: Synd-3 (N-Syndecan) extracellular domain

gccccgcgcgctgctgagccgtccttgccggcacgssgatgcccgaggagctgcggcgcc  
tcgcggtgctgctgctgctgctcagcgccccgcgcagcgctggcctcagccgtggcgcaatg  
agaactacgagaggccggtggacctggagggctctggggatgatgatccctttggggacg  
atgaactggatgacatctactcggggtccgggtcaggctattttgagcaggagtcagggt  
tgagacagcgggtcagcctcaccacggacacgtccgtcccactgcccaccacgggtggccg  
tgctgcctgtcaccttggtgcagcccatggcaacaccctttgagctgttccccacagagg  
acacgtcccctgagcaaacaaccagcgtcttgtatatccccaagataacagaagcaccag  
tgatccccagctggaaaacaaccaccgcccagttaccactgccagtgactccccagttacca  
cctccaccaccaccaccacgggtgctaccaccaccacaaccaccaccaccatcagcacca  
ctgtggccacctccaagcccaccactaccagagggttcctgccccctttgtcaccaagg  
cagccaccaccggggccaccaccctggagacgcccaccaccctccatccctgaaaccagtg  
tcctgacagagggtgaccacatcacggcttggtcccctccagcacagccaagccgagggtccc  
tgccaaaaccaagcacttccaggactgcagaacccacggaaaaaagcactgccttgcctt  
ccagccccaccacgctgccaccacagaagccccccagggtggagccaggggagttgacga  
cagtcctcgacagtgacctggaagtcccaaccagtagtggtcccccagcggggacttcgaga  
tccaggaggaggaggagacaactcgtcctgagctgggcaatgaggtggtggcagtggtga  
caccaccagcagcaccgggggtgggcaagaatgcagagccgggggtcatcgacaacacaa  
tagagtcgggcagctcgggtgctcagctccccagaaaaacatcctggagaggaaggaa

### Reference:

ACCESSION M84910  
LOCUS CHKSNDCPRO 1372 bp mRNA VRT 29-NOV-1995  
DEFINITION Chicken syndecan-3 proteoglycan mRNA, complete cds.  
ACCESSION M84910  
NID g1017461  
KEYWORDS syndecan-3 proteoglycan.  
SOURCE Gallus gallus 4-6 day and 10 day forelimb and whole embryo cDNA to mRNA.  
ORGANISM Gallus gallus  
Eukaryotae; mitochondrial eukaryotes; Metazoa; Chordata;  
Vertebrata; Archosauria; Aves; Neognathae; Galliformes;  
Phasianidae; Phasianinae; Gallus.  
REFERENCE 1 (bases 1 to 1372)  
AUTHORS Gould,S.E., Upholt,W.B. and Kosher,R.A.  
TITLE Syndecan 3: a member of the syndecan family of  
membrane-intercalated proteoglycans that is expressed in high  
amounts at the onset of chicken limb cartilage differentiation  
JOURNAL Proc. Natl. Acad. Sci. U.S.A. 89 (8), 3271-3275 (1992)  
MEDLINE 92228766  
REFERENCE 2 (bases 1 to 1372)  
AUTHORS Gould,S.E., Upholt,W.B. and Kosher,R.A.  
TITLE Characterization of chicken syndecan-3 as a heparan sulfate  
proteoglycan and its expression during embryogenesis  
JOURNAL Dev. Biol. 168 (2), 438-451 (1995)

Fig. 5: Syndecan-4 extra-cellular domain from rat: M81786

atggc gcctgtctgc ctgtttgcgc cgtgtctgct gttgctcctc  
ggaggtttcc ccgtcgcccc aggcgagtcg attcgagaga ctgagggtcat agacccccag  
gacctcctgg aaggcagata cttctctgga gccctcccgg acgatgaaga cgctggggggc  
cttgagcagg actctgactt tgagctgtcg gggtccggag atctagatga cacggaggag  
cccaggacct tccctgaggt gatttcaccc ttggtgccac tagataacca catccccgag  
aatgcccagc ctggcatccg tgtcccctca gagcccaagg aactggaaga gaatgaggtc  
attcccaaaa ggggtcccctc cgacgtgggg gatgacgatg tgtccaacaa agtgtccatg  
tccagcactt cccagggcag caacattttt gaaagaactg ag

Reference:

ACCESSION M81786  
LOCUS RATRYUDOCA 2452 bp mRNA ROD 16-JUL-1992  
DEFINITION Rattus norvegicus ryudocan mRNA, complete cds.  
ACCESSION M81786  
NID g206822  
KEYWORDS ryudocan.  
SOURCE Rattus norvegicus Epididymal fat pad cDNA to mRNA.  
ORGANISM Rattus norvegicus  
Eukaryotae; mitochondrial eukaryotes; Metazoa; Chordata;  
Vertebrata; Eutheria; Rodentia; Sciurognathi; Myomorpha; Muridae;  
Murinae; Rattus.  
REFERENCE 1 (bases 1 to 2452)  
AUTHORS Kojima,T., Shworak,N.W. and Rosenberg,R.D.  
TITLE Molecular cloning and expression of two distinct cDNA-encoding  
heparan sulfate proteoglycan core proteins from a rat endothelial  
cell line  
JOURNAL J. Biol. Chem. 267, 4870-4877 (1992)

3362090-91634150

Fig. 6: Glypican-1 (rat) extracellular Domain:

atggag

```
ctccggggccc gaggctggtg gctgctgtgc gcggccgcgc cgctagtgcg ctgcgcccgc
ggggacccccg ccagcaagag ccggagctgc agcgaagtcc gccagatcta cggggctaag
ggcttttagcc tgagcgacgt gccccaggca gagatctcgg gagagcacct gcggatctgc
ccccagggct acacctgctg caccagttag atggaggaga acctggccaa ccacagccgg
atggagctgg agaccgcact ccacgacagc agccgtgccc tgcaggctac actggccacc
cagctgcatg gcatcgatga ccacttccag cgcctgctga atgactcgga gcgtacactg
caggatgctt ttcccggggc ctttggggac ctgtacacgc agaacactcg ggccttccgg
gacctgtatg ctgagctgcg tctctactac cgagggggcca acctacacct tgaggagaca
ctggccgagt tctgggcacg gctgctggag cgtctcttca agcagctgca cccccagctt
ctgctgcccc atgactatct ggactgcctg ggcaagcagg cagaggcact gcggccgttt
ggggatgccc ctcgagaact gcgcctgagg gccaccctgt cttttgtggc ggcacgatec
tttgtgcagg gcctgggtgt ggccagttag gtagtccgaa aggtggccca ggttcctctg
gccccagaat gttctcgggc tgtcatgaag ttggtctact gtgcccattg ccggggagtc
cctggtgccc ggccctgtcc cgactattgc cgaaatgtgc tcaaaggctg ccttgccaac
caggccgacc tggatgccga gtggagggaac ctcttgact ccattggtgct catcactgac
aagttctggg gcccgctcggg tgcggagaat gtcattggca gtgtgcatat gtggctggcg
gaggccatca acgccctcca ggacaacaag gacacactca cagctaaggc catccagggc
tgcggaacc ccaagggtcaa tccccatggc tctgggacct aggagaagcg tgcctgtggc
aaactggcac tgcaggagaa gtctctccaca ggtactctgg aaaagctggc ctctgaggcc
aaggcccagc tccgagacat tcaggactac tggatcagcc tcccagggac actgtgtagt
gagaagatgg ccatgagtcg tgccagcgat gaccgctgct ggaatgggat ttccaagggc
cggtacctac ctgaggtgat ggggtgatggg ctggccaacc agatcaacaa ccctgaagtg
gaggtggaca tcaccaagcc ggatatgacc atccggcagc agatcatgca gctcaagatc
atgaccaacc gtttacgtgg cgcctacggt ggcaatgatg tggacttcca ggatgccagt
gatgacggca gtggctccgg cagcgggtggc ggatgcccag atgacgcctg tggccggagg
gtcagcaaga agagctccag ctcccgagcc cccttgaccc atgccctccc cggtttgtca
gaacaggagg gacagaagac ctcg
```

Protein sequence:

```
MELRARGWLLCAAALVACARGDPASKSRSCSEVRQIYGAKGF
SLSDVPQAEISGEHLRICPQGYTCCTSEMEENLANHSRMELETALHDSSRALQATLAT
QLHGIDDFQRLNDSERTLQDAFPGAFGDLYTQNTAFRDLYAELRLYYRGANLHLE
ETLAEFWARLLERLFKQLHPQLLLPDDYLDCLGKQAEALRPFDPAPRELRLRATRAFV
AARSFVQGLGVASDVVRKVAQVPLAPECSRVMKLVYCAHCRGVPGARPCPDYCRNVL
KGCLANQADLDAEWRNLLDSMVLITDKFWGPGSGAENVIGSVHMLAEAINALQDNKDT
LTAKVIQGCNPKVNPBGSGPEEKRRRGKLLAQEKSSSTGTLEKLVSEAKAQLRDIQDY
WISLPGLTLCSEKMAMSPASDDRCWNGISKGRYLPEVMGDGLANQINNPEVEVDITKPD
MTIRQQIMQLKIMTNRLRGAYGGNDVDFQDASDDGSGSGSGGGCPDDACGRRVSKKSS
SSRTPLTHALPGLSEQEGQKTS
```

Reference:

ACCESSION L34067  
NID g506416  
KEYWORDS glypican.  
SOURCE Rattus norvegicus (strain New England Deconess Hospital) cDNA to mRNA.  
ORGANISM Rattus norvegicus  
Eukaryotae; mitochondrial eukaryotes; Metazoa; Chordata;  
Vertebrata; Eutheria; Rodentia; Sciurognathi; Myomorpha; Muridae;  
Murinae; Rattus.  
REFERENCE 1 (bases 1 to 1737)  
AUTHORS Litwack, E.D., Stipp, C.S., Kumbasar, A. and Lander, A.D.  
TITLE Neuronal expression of glypican, a cell-surface  
glycosylphosphatidylinositol-anchored heparan sulfate proteoglycan,  
in the adult rat nervous system  
JOURNAL J. Neurosci. 14, 3713-3724 (1994)

Fig.7 : Syndecan-1 (rat) transmembrane domain: M81785

gtgct gggaggtgtc attgctggag gcctggtggg  
cctcatcttt gctgtgtgcc tgggtggcttt catgctatac

Reference:

ACCESSION M81785  
LOCUS RATSYNDECA 2396 bp mRNA ROD 16-JUL-1992  
DEFINITION Rattus norvegicus syndecan mRNA, complete cds.  
ACCESSION M81785  
NID g207140  
KEYWORDS syndecan.  
SOURCE Rattus norvegicus Epididymal fat pad cDNA to mRNA.  
ORGANISM Rattus norvegicus  
Eukaryotae; mitochondrial eukaryotes; Metazoa; Chordata;  
Vertebrata; Eutheria; Rodentia; Sciurognathi; Myomorpha; Muridae;  
Murinae; Rattus.  
REFERENCE 1 (bases 1 to 2396)  
AUTHORS Kojima,T., Shworak,N.W. and Rosenberg,R.D.  
TITLE Molecular cloning and expression of two distinct cDNA-encoding  
heparan sulfate proteoglycan core proteins from a rat endothelial  
cell line  
JOURNAL J. Biol. Chem. 267, 4870-4877 (1992)

004430-0909

Fig. 8 : Syndecan-2 (human) transmembrane domain (J 04621):

gtcctagcag ctgtcattgc tgggtggagtt attggctttc tctttgcaat ttttcttata  
ctgctgttgg tg

protein sequence:

VLA AVIAGGVIGFLFAIFLILLV

reference:

ACCESSION J04621

LOCUS HUMHSPGC 3414 bp mRNA PRI 08-NOV-1994

DEFINITION Human heparan sulfate proteoglycan (HSPG) core protein, 3' end.

ACCESSION J04621

NID g184428

KEYWORDS core protein; heparan sulfate proteoglycan.

SOURCE Human fetal lung fibroblast, cDNA to mRNA, clone 48K5.

ORGANISM Homo sapiens

Eukaryotae; mitochondrial eukaryotes; Metazoa; Chordata;

Vertebrata; Eutheria; Primates; Catarrhini; Hominidae; Homo.

REFERENCE 1 (bases 1 to 3414)

AUTHORS Marynen,P., Zhang,J., Cassiman,J.J., Van den Berghe,H. and David,G.

TITLE Partial primary structure of the 48- and 90-kilodalton core proteins of cell surface-associated heparan sulfate proteoglycans of lung fibroblasts. Prediction of an integral membrane domain and evidence for multiple distinct core proteins at the cell surface of human lung fibroblasts

JOURNAL J. Biol. Chem. 264 (12), 7017-7024 (1989)

00141854780

## Fig. 9 : Synd-3 (N-Syndecan) chicken Transmembrane domain

gtgttgatagctgtgattgtcggcggtgtggtgggagccctctttgctgccttccttggtca  
tgctgctcatctac

### Reference:

ACCESSION M84910  
LOCUS CHKSNDPCPRO 1372 bp mRNA VRT 29-NOV-1995  
DEFINITION Chicken syndecan-3 proteoglycan mRNA, complete cds.  
ACCESSION M84910  
NID g1017461  
KEYWORDS syndecan-3 proteoglycan.  
SOURCE Gallus gallus 4-6 day and 10 day forelimb and whole embryo cDNA to mRNA.  
ORGANISM Gallus gallus  
Eukaryotae; mitochondrial eukaryotes; Metazoa; Chordata;  
Vertebrata; Archosauria; Aves; Neognathae; Galliformes;  
Phasianidae; Phasianinae; Gallus.  
REFERENCE 1 (bases 1 to 1372)  
AUTHORS Gould,S.E., Upholt,W.B. and Kosher,R.A.  
TITLE Syndecan 3: a member of the syndecan family of  
membrane-intercalated proteoglycans that is expressed in high  
amounts at the onset of chicken limb cartilage differentiation  
JOURNAL Proc. Natl. Acad. Sci. U.S.A. 89 (8), 3271-3275 (1992)  
MEDLINE 92228766  
REFERENCE 2 (bases 1 to 1372)  
AUTHORS Gould,S.E., Upholt,W.B. and Kosher,R.A.  
TITLE Characterization of chicken syndecan-3 as a heparan sulfate  
proteoglycan and its expression during embryogenesis  
JOURNAL Dev. Biol. 168 (2), 438-451 (1995)

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Figure 10 : Syndecan-4 transmembrane domain from rat: M81786

gtcttggc agctctgatt gtgggcggcg tagtgggcat cctcttcgcc gttttcctga  
tcctgctgct ggtgtac

Reference:

ACCESSION M81786  
LOCUS RATRYUDOCA 2452 bp mRNA ROD 16-JUL-1992  
DEFINITION Rattus norvegicus ryudocan mRNA, complete cds.  
ACCESSION M81786  
NID g206822  
KEYWORDS ryudocan.  
SOURCE Rattus norvegicus Epididymal fat pad cDNA to mRNA.  
ORGANISM Rattus norvegicus  
Eukaryotae; mitochondrial eukaryotes; Metazoa; Chordata;  
Vertebrata; Eutheria; Rodentia; Sciurognathi; Myomorpha; Muridae;  
Murinae; Rattus.  
REFERENCE 1 (bases 1 to 2452)  
AUTHORS Kojima,T., Shworak,N.W. and Rosenberg,R.D.  
TITLE Molecular cloning and expression of two distinct cDNA-encoding  
heparan sulfate proteoglycan core proteins from a rat endothelial  
cell line  
JOURNAL J. Biol. Chem. 267, 4870-4877 (1992)

362050-974190

1. The first step in the process is to identify the problem or issue that needs to be addressed. This involves gathering information and understanding the context of the problem.

2. Once the problem is identified, the next step is to define the objectives and goals of the project. This helps to clarify what needs to be achieved and provides a clear direction for the team.

3. The third step is to develop a plan or strategy to address the problem. This involves breaking down the problem into smaller, manageable tasks and determining the resources needed to complete each task.

4. The fourth step is to implement the plan. This involves putting the strategy into action and monitoring progress regularly to ensure that the project is on track.

5. The final step is to evaluate the results of the project. This involves comparing the actual outcomes with the objectives and goals to determine the effectiveness of the project and identify areas for improvement.

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5. The final step is to evaluate the results of the project. This involves comparing the actual outcomes with the objectives and goals to determine the effectiveness of the project and identify areas for improvement.

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2. Once the problem is identified, the next step is to define the objectives and goals of the project. This helps to clarify what needs to be achieved and provides a clear direction for the team.

3. The third step is to develop a plan or strategy to address the problem. This involves breaking down the problem into smaller, manageable tasks and determining the resources needed to complete each task.

4. The fourth step is to implement the plan. This involves putting the strategy into action and monitoring progress regularly to ensure that the project is on track.

5. The final step is to evaluate the results of the project. This involves comparing the actual outcomes with the objectives and goals to determine the effectiveness of the project and identify areas for improvement.

1. The first step in the process is to identify the problem or issue that needs to be addressed. This involves gathering information and understanding the context of the problem.

2. Once the problem is identified, the next step is to define the objectives and goals of the project. This helps to clarify what needs to be achieved and provides a clear direction for the team.

3. The third step is to develop a plan or strategy to address the problem. This involves breaking down the problem into smaller, manageable tasks and determining the resources needed to complete each task.

4. The fourth step is to implement the plan. This involves putting the strategy into action and monitoring progress regularly to ensure that the project is on track.

5. The final step is to evaluate the results of the project. This involves assessing the outcomes against the objectives and goals and identifying any areas for improvement or further action.

Figure 12 : Perlecan (human) transmembrane domain #M85289

tcgcgacactgctcatcccag  
ccatcacgactgctgacgccggcttctacctctgcggtggccaccagccctgcaggcactg  
cc

Reference:

LOCUS HUMHSPG2B 14327 bp mRNA PRI 08-NOV-1994  
DEFINITION Human heparan sulfate proteoglycan (HSPG2) mRNA, complete cds.  
ACCESSION M85289  
NID g184426  
KEYWORDS HSPG2 gene; heparan sulfate proteoglycan.  
SOURCE Homo sapiens skin; colon cDNA to mRNA.  
ORGANISM Homo sapiens  
Eukaryotae; mitochondrial eukaryotes; Metazoa; Chordata;  
Vertebrata; Eutheria; Primates; Catarrhini; Hominidae; Homo.  
REFERENCE 1 (bases 1 to 14327)  
AUTHORS Dodge,G.R., Kovalszky,I., Chu,M.L., Hassell,J.R., McBride,O.W.,  
Yi,H.F. and Iozzo,R.V.  
TITLE Heparan sulfate proteoglycan of human colon: partial molecular  
cloning, cellular expression, and mapping of the gene (HSPG2) to  
the short arm of human chromosome 1  
JOURNAL Genomics 10 (3), 673-680 (1991)  
MEDLINE 91365376  
REFERENCE 2 (bases 1 to 14327)  
AUTHORS Murdoch,A.D., Dodge,G.R., Cohen,I., Tuan,R.S. and Iozzo,R.V.  
TITLE Primary structure of the human heparan sulfate proteoglycan from  
basement membrane (HSPG2/perlecan). A chimeric molecule with  
multiple domains homologous to the low density lipoprotein  
receptor, laminin, neural cell adhesion molecules, and epidermal  
growth factor  
JOURNAL J. Biol. Chem. 267 (12), 8544-8557 (1992)

0044536-00000000

Figure 13 : Syndecan-4 cytoplasmic domain from rat: M81786

Cgc atgaagaaga aggatgaagg cagttacgac ttgggcaaga aacccatcta caaaaaagcc  
cccaccaacg agttctacgc atga

Reference:

ACCESSION M81786  
LOCUS RATRYUDOCA 2452 bp mRNA ROD 16-JUL-1992  
DEFINITION Rattus norvegicus ryudocan mRNA, complete cds.  
ACCESSION M81786  
NID g206822  
KEYWORDS ryudocan.  
SOURCE Rattus norvegicus Epididymal fat pad cDNA to mRNA.  
ORGANISM Rattus norvegicus  
Eukaryotae; mitochondrial eukaryotes; Metazoa; Chordata;  
Vertebrata; Eutheria; Rodentia; Sciurognathi; Myomorpha; Muridae;  
Murinae; Rattus.  
REFERENCE 1 (bases 1 to 2452)  
AUTHORS Kojima,T., Shworak,N.W. and Rosenberg,R.D.  
TITLE Molecular cloning and expression of two distinct cDNA-encoding  
heparan sulfate proteoglycan core proteins from a rat endothelial  
cell line  
JOURNAL J. Biol. Chem. 267, 4870-4877 (1992)

094454160

091459E-090293  
092050-0954150

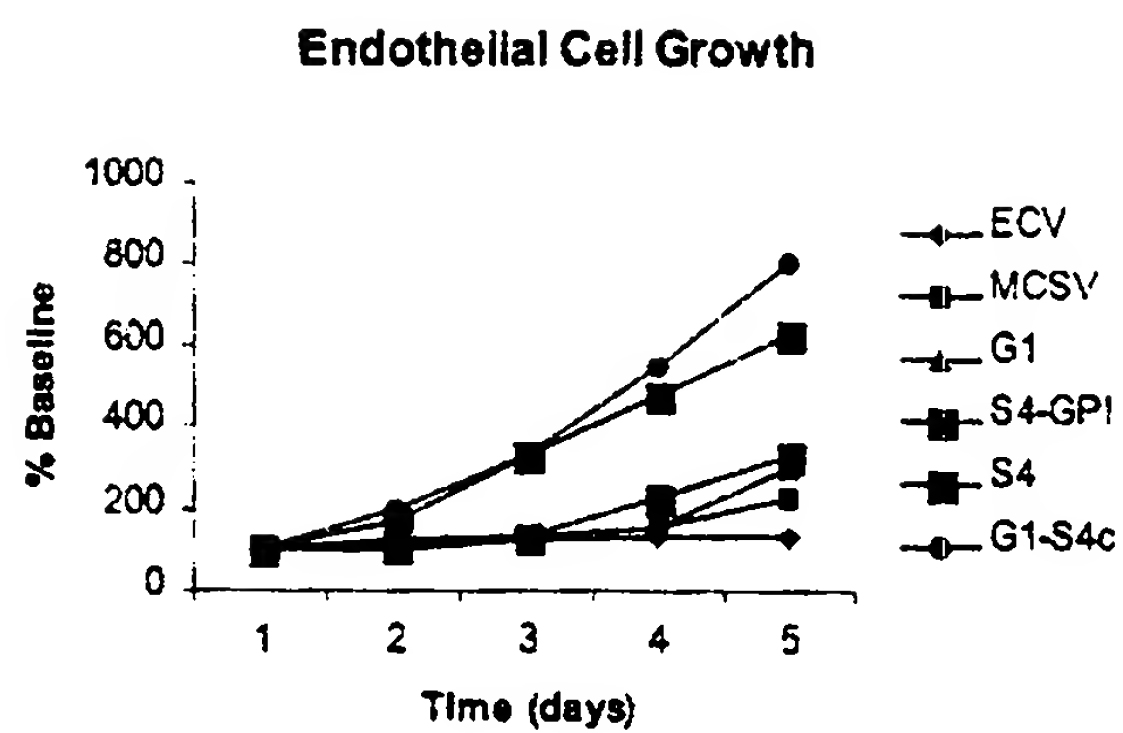


Fig. 14

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Applicant or Patentee: M. Simons, R. Volk & A. Horowitz Attorney's  
Serial or Patent No.: \_\_\_\_\_ Docket No.: BIS-039  
Filed or Issued: \_\_\_\_\_  
For: "STIMULATION OF ANGIOGENESIS VIA ENHANCED ENDOTHELIAL EXPRESSION OF  
SYNDECAN-4 CORE PROTEINS"

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY  
STATUS (37 CFR 1.9 (f) and 1.27 (b)) — INDEPENDENT INVENTOR

As a below named inventor, I hereby declare that I qualify as an independent inventor as defined in 37 CFR 1.9 (c) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, to the Patent and Trademark Office with regard to the invention entitled as above described in

☒ the specification filed herewith  
☐ application serial no. \_\_\_\_\_, filed \_\_\_\_\_  
☐ patent no. \_\_\_\_\_, issued \_\_\_\_\_

I have not assigned, granted, conveyed or licensed and am under no obligation under contract or law to assign, grant, convey or license, any rights in the invention to any person who could not be classified as an independent inventor under 37 CFR 1.9 (c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 CFR 1.9 (d) or a nonprofit organization under 37 CFR 1.9 (e).

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

☐ no such person, concern, or organization  
☒ persons, concerns or organizations listed below\*

\*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

FULL NAME Beth Israel Deaconess Medical Center  
ADDRESS 330 Brookline Avenue, Boston, MA 02215  
☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☒ NONPROFIT ORGANIZATION  
FULL NAME \*\*\*\*\*  
ADDRESS \*\*\*\*\*  
☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION  
FULL NAME \*\*\*\*\*  
ADDRESS \*\*\*\*\*  
☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28 (b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Michael Simons Rudiger Volk Arie Horowitz  
NAME OF INVENTOR NAME OF INVENTOR NAME OF INVENTOR

\_\_\_\_\_  
Signature of Inventor Signature of Inventor Signature of Inventor  
\_\_\_\_\_  
Date Date Date

Attorney's Docket No. BIS-039

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled "STIMULATION OF ANGIOGENESIS VIA ENHANCED ENDOTHELIAL EXPRESSION OF SYNDECAN-4 CORE PROTEINS",

the specification of which: (check one)

XXXXX is attached hereto:

\_\_\_\_\_ was filed on \_\_\_\_\_ as Application Serial No. \_\_\_\_\_:

\_\_\_\_\_ was amended on \_\_\_\_\_ (if applicable):

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign applications for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that on which priority is claimed:

PRIOR FOREIGN APPLICATION(S)

Priority claimed

.....NONE.....  
(Number) (Country) (Day/month/year/filed) Yes No

.....NONE.....  
(Number) (Country) (Day/month/year/filed) Yes No



I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

.....NONE.....  
(Application Serial No.) (Filing Date) (Status)

.....NONE.....  
(Application Serial No.) (Filing Date) (Status)

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POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney to prosecute this application and transact all business in the Patent and Trademark Office connected therewith; and, in addition, to act as Agent on my behalf before the competent International Authorities and before the National Authorities for any designated countries in connection with any and all international applications filed or to be filed by the undersigned.

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Registration Number 29,693

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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